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VOLUME TWENTY-THIRD

WITH ONE HUNDRED AND FIFTEEN PLATES AND THIRTY-SEVEN  
FIGURES IN THE TEXT

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## KLOSSIELLA INFECTION OF THE GUINEA PIG.

By LOUISE PEARCE, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

PLATES 63 TO 70.

(Received for publication, January 31, 1916.)

In studying the lesions produced by arsenical compounds in the kidneys of different species of animals, certain parasites were observed in the renal tubules of guinea pigs, which strikingly resembled the coccidium, *Klossiella muris*, observed and described by Smith and Johnson<sup>1,2</sup> in the kidneys of the mouse. As we believed the guinea pig to be relatively free from spontaneous renal lesions, a search through the literature was made to ascertain if renal parasites of the guinea pig had been previously described. At the same time the study of a large number of guinea pig kidneys was undertaken, in order to determine the relative frequency of this parasite as well as to obtain information concerning the lesions which tend to be associated with it.

Seidelin<sup>3</sup> in 1914 describes a parasite which occurred in the kidneys of two guinea pigs from Nigeria, and which bears such strong resemblance to *Klossiella muris* in some of its stages that he considers that both parasites "must be regarded as belonging to one and the same genus, whilst the question of their specific identity or otherwise cannot at the present time be finally decided." He further states that the parasite does not appear to be a common one in West African guinea pigs, as he found only one case among about twenty guinea pigs of the Yaba series. Later he found identical parasites in sections of a kidney of another guinea pig from the same locality. He found no similar instance in guinea pigs procured in Liverpool.

<sup>1</sup> Smith, T., *Jour. Comp. Med. and Surg.*, 1889, x, 211.

<sup>2</sup> Smith, T., and Johnson, H. P., *Jour. Exper. Med.*, 1914, vi, 303.

<sup>3</sup> Seidelin, H., *Ann. Trop. Med. and Parasit.*, 1914-15, viii, 553.

Seidelin describes some of the stages of the parasite detected by him and suggests a probable life cycle. There is undoubtedly a close resemblance between this parasite and *Klossiella muris*, and again between both these parasites and the one about to be described. On the other hand, the question of the identity of Seidelin's parasite and the one we have observed will be discussed later on in this paper. There are certainly points of similarity between the two, as well as some points of difference.

It may be stated here that we have found the infection to be by no means uncommon in kidneys of guinea pigs. In sixty guinea pigs examined for renal parasites, twelve were found to be infected. In other words, in our experiments with guinea pigs we found that we must reckon upon at least 20 per cent of the animals from our sources being infected with this renal parasite.

The material examined consisted of kidney sections of sixty adult guinea pigs from two sources, one in Philadelphia, the other in New Brunswick, New Jersey. These pigs were either perfectly normal animals or were animals which had been used for toxicological experiments with various arsenical compounds and had survived the use of the drug not more than 24 hours. This arbitrary time limit was set in the choice of material in order that any chronic pathological process observed might not be confused with the drug action, since within this time (24 hours) no extensive proliferative change in the kidney can be attributed to the action of these compounds. The kidney tissue was fixed in Zenker's fluid and imbedded in paraffin. Sections were stained with hematoxylin and eosin, methylene blue and eosin, and Giemsa's stain. In addition, frozen sections were made from tissue fixed in 10 per cent formalin and stained with hematoxylin and eosin.

#### *Description of the Parasite.*

Various stages of the parasite have been seen in the kidneys of guinea pigs and in order to facilitate an interpretation of its probable life cycle, a certain sequence of description will be observed, beginning with the stage or form most frequently encountered.

*Sporoblast Cycle.*—The form most frequently seen is a small round or ovoid body measuring 7 to 8  $\mu$  in diameter. It stains a very light



bluish pink with hematoxylin and eosin, and contains numerous small dark blue masses of chromatin scattered through the cytoplasm in no definite arrangement (Figs. 1 and 2). The cytoplasm is somewhat granular and slightly, if at all refractile. The chromatin is of two sizes, larger irregular masses and tiny pin points, both taking a dark blue stain with hematoxylin and methylene blue. Usually several of these parasites are found free in the lumen of the renal tubules and sometimes the lumen appears to be completely blocked with them. They are most frequently seen in the straight tubules of the inner half of the cortex, but are also found somewhat less numerous in the convoluted tubules and in the tubules of the upper medulla. Occasionally one or two may be found in the capsular space of the glomerulus. This stage of the parasite is apparently very similar to the so called "daughter sporoblast" stage of *Klossiella muris* described by Smith and Johnson.

In certain sections it has been possible to make out a confining cell membrane surrounding several of these bodies, as in Fig. 2. The membrane is all that remains of the epithelial cell that originally contained the developing parasites. As they grow and increase in size the host cell must necessarily enlarge until it becomes flask-shaped and bulges out into the lumen of the tubule. The parasites occupy the broad distal portion of the containing cell and the nucleus is usually crowded inwards and often to one corner of the cell. It frequently appears flattened, compressed, or shrunken, and eventually it disappears, apparently just preceding or about the time of rupture of the cell. The cytoplasm of the host cell gradually becomes more and more scanty and granular and finally all that remains of the cell is its enormously distended cell wall, which is ruptured by the escaping parasites. It is somewhat difficult to determine just how many parasites develop in the epithelial cell, as we have had no opportunity of examining any material except cross sections of kidney tissue. However, there are probably at least ten to twelve.

Each of these parasites next undergoes a division or segmentation into eight to twelve bodies (Fig. 3). These bodies are falciform or banana-shaped and are extremely small, measuring 1 by 4 to 5  $\mu$ , and are surrounded by a distinct membrane. Their cytoplasm is clear and non-granular, pink (hematoxylin and eosin, and Giemsa's

stain), and each body contains a dark blue dot of chromatin. We believe that these tiny fusiform bodies are similar to the form designated as the sporozoites of *Klossiella muris*. The further history of these bodies is entirely conjectural and will be referred to later in the discussion.

Not uncommonly one will find in heavily infected kidneys a form (Fig. 4) which is evidently the precursor of the two stages just described. This form is comparatively large, and may measure 17 by 22  $\mu$ ; it has the same appearance and staining characteristics as the smaller more usual forms first described (Figs. 1 and 2). A breaking up of this large mass into several constituent smaller bodies results in a picture similar to Fig. 2. In studying various infected kidneys, we have seen several microscopic fields in which there were all gradations of this division (Fig. 6), so that we are inclined to regard this large parasitic mass as corresponding to the so called "mother sporoblast" stage of *Klossiella muris*.

*Ring Form.*—In several of the sections of heavily infected kidneys we have seen a stage of the parasite which we have called the "ring," or "annular" form (Figs. 7 and 8). It is always within an epithelial cell and the cell itself is enlarged and may protrude into the lumen of the tubule to the extent of nearly occluding it, as in Fig. 8. This form measures 18 to 20  $\mu$  in diameter and consists of a series of twelve to eighteen definite divisions or segments arranged in a circle or ring. Each division is extremely small, ovoid in shape, and measures 3 by 5 to 7  $\mu$ . They are smaller than the more frequently encountered forms seen in Figs. 1, 2, 3, and 4 (the daughter sporoblasts), and in appearance are totally different. The cytoplasm is refractile and stains practically not at all, or at most a very pale pink with hematoxylin and eosin, and Giemsa's stain. Each segment contains one or two chromatin dots. In some instances the two chromatin dots appear to be fusing together; in others they are distinctly separate from one another. In other instances the one chromatin dot is elliptical in shape, and it is possible that the finding of two chromatin dots is due to the level of the section. In the center of the ring form is a small amount of a granular refractile pale pink staining material which is apparently a residuum of the cytoplasm of the surrounding ring segments (Figs. 7 and 8).

In Fig. 8 there is a typical ring body and another intracellular form which we think is also a ring body seen from the outside. In other words, a ring body is a section through such a hollow sphere as is seen in Figs. 9 and 10. Here there are eighteen small segments or divisions, each of which is similar in appearance and staining reactions to the segments of the ring form.

The ring segments are not only considerably larger than the final divisions of the sporoblast cycle, the sporozoites (Fig. 5), but are also of a totally different shape and appearance. Moreover, the immediate precursor stage, the ring form, is still enclosed in an epithelial cell, while the precursor stage of the falciform bodies shown in Fig. 5 is not necessarily intracellular. Indeed, when the division into the tiny falciform bodies or sporozoites occurs, the daughter sporoblasts are probably always extracellular and free in the lumen of the tubule. In addition, the sporozoites are contained in a very distinct spore, having a definite membrane, while there is no such structure surrounding the segments of the ring form. When the ring divides, the resulting segments are all apparently set free in the remains of the epithelial host cell and eventually in the lumen of the tubule when this cell ruptures. The ring segments seem to be identical with still another stage of the parasite about to be described and may represent its earliest and youngest form.

*Hyaline Forms.*—Figs. 11 to 16 illustrate a single stage of the parasite which from its appearance and staining character is evidently one of the segments of the ring form. It is very small, measuring from 5 to 8  $\mu$  in length and 3 to 5  $\mu$  in breadth, and is oval or ovoid in shape. The cytoplasm is non-granular, extremely refractile, hyaline, and stains pink with hematoxylin and eosin, or Giemsa's stain. It contains a relatively large clear-cut mass of dark blue staining chromatin placed towards one end of the parasite. At the opposite pole a small clear non-staining area may be seen, as in Fig. 12. In the section from which Fig. 11 was taken this clear area is visible, but it is very small and barely shows in the photograph. This stage measures 3 by 5.5 to 7  $\mu$ . It may be extra- or intracellular, as shown in the illustrations. In Fig. 12 it apparently is about to enter an epithelial cell of a renal tubule. In Fig. 11 it is clearly intracellular and surrounded by a vacuole. The nucleus of the host

cell is immediately below the parasite. The epithelial cell opposite the parasite on the other side of the renal tubule contains an ovoid inclusion which is probably a similar parasitic body, although the mass of chromatin is not seen in the section. The cytoplasm of the two cellular inclusions is identical.

In Fig. 13 another intracellular parasite is seen and here the chromatin has divided into two distinct parts. The parasite itself measures 5 by 7  $\mu$ . The host cell has become enormously enlarged and protrudes into the lumen of the tubule. Fig. 14 shows a further division of the chromatin into four distinct segments. This parasite is also intracellular, but in order to bring the chromatin into focus for the photograph, the epithelial cell outlines are not seen. Just below this parasite is another smaller intracellular form similar to those shown in Figs. 11 and 12, with only one chromatin mass.

*Schizogonic Cycle.*—Fig. 17 illustrates a spherical form which we are inclined to believe represents the schizogony of the parasite. It is comparatively large—measuring 22  $\mu$  in diameter, and is composed of a large number (thirty to forty) of tiny fusiform bodies or merozoites enclosed in a rather indistinct and apparently very thin membrane. It does not appear to be enclosed in an epithelial cell, but is extracellular and free in the lumen of the renal tubule. The merozoites in longitudinal section measure 1.5 by 7 to 8  $\mu$ . Their cytoplasm stains pink with hematoxylin and eosin, and each contains a tiny dot of chromatin. These small falciform bodies are extremely similar to the sporozoites described above. The general appearance of this large form suggests its similarity, if not identity with the glomerular body of *Klossiella muris*, which Smith and Johnson interpret as the schizogony of the mouse parasite. We have not found this form in the capsular space of the glomeruli but in the convoluted tubules of the first order. It is not a common stage and we have found it in only one kidney.

*Pathological Changes in the Kidneys of Guinea Pigs Associated with the Parasitic Infection.*

The kidneys of guinea pigs infected with the parasite show certain pathological changes of a chronic nature which we are inclined to attribute to the presence of the organism. They have been found

in all cases in which the parasite has been seen, and in several instances where these lesions were observed the infection was very light, and the parasite was found only after a thorough search.

The lesions which we think are caused by the parasite consist in an irregular accumulation of fibroblasts and small round cells about the base of some of the glomeruli. Both the distribution and arrangement of this infiltration are very irregular (Figs. 18 to 21). In a single microscopic field one or two glomeruli may be affected in this manner, while the adjacent glomeruli are normal in appearance. Moreover, the extent of the infiltration varies considerably. Some glomeruli have only a slight accumulation of round cells about their base, others are almost obliterated, as in Figs. 18, 20, and 21. Usually there seem to be relatively more cells of the small round mononuclear type than fibroblasts. The fibroblasts themselves are apparently not young cells.

These cellular accumulations seem to be fairly well confined to the immediate vicinity of the glomeruli. In certain instances, however, the round cells and fibroblasts extend outward to a limited degree into the labyrinthine tissue and along the medullary rays between the tubules, but in these cases the connection between this extension and the accumulation of similar cells about the neighboring glomeruli can be easily traced.

There seems to be no reaction of the kidney tissue in the immediate vicinity of the parasite itself, that is, in the inner half of the cortex where we have found the parasite to be most numerous. Here one may see half a dozen consecutive tubules filled with parasites and eight or ten epithelial cells containing ring forms, yet there is apparently no abnormality in the immediate interstitial connective tissue. The glomeruli, however, just above these infected tubules show a more or less extensive infiltration of round cells and fibroblasts. The portion of medulla just below the infected tubule shows no appreciable change. No gross changes in the kidneys referable to the renal parasite have been noted.

#### DISCUSSION.

The description of the various stages of the parasite found in the kidney of the guinea pig has been arranged so as to relate, tentatively at least, those forms which seem to belong to the same cycles

of development. Certainly two different cycles of development have been observed. The exact interpretation of the cycles must be more or less hypothetical, since there is an obvious lack of knowledge of all the stages in the evolution of the parasite.

The first cycle, described under the sporozoites is similar to the so called sporoblast stage of *Klossiella muris* and is evidently similar to the stage described by Seidelin. However, we have never seen more than twelve daughter sporoblasts, and usually only eight to ten resulting from the first division of the mother or pan sporoblast form, while Seidelin gives sixteen to twenty as the probable number. This discrepancy, if the two parasites are identical, can probably only be settled by the study of fresh material or an extensive series of sections. In the second division, into sporozoites, we have never observed more than twelve, each sporozoite measuring 1 by 4 to 5  $\mu$ . Seidelin describes thirty sporozoites, each measuring 1.5 by 8  $\mu$ . If this difference in the number of sporozoites continues to hold after the study of fresh tissue, we shall be inclined to believe that the two parasites belong to different species.

That this cycle of the parasitic development represents the sporoblast phase is strongly suggested by the fact that the sporozoites are confined in what is apparently a typical spore. We have never seen these spore-like bodies breaking up in the lumen of the kidney tubules, and it is probable that the spores containing the sporozoites are excreted in the urine, which is afterwards swallowed by the same or other guinea pigs, and that the spore membrane is digested away by the gastric juice, thus freeing the sporozoites. The extremely small size and fusiform shape of these tiny bodies would doubtless enable them to pass through the gastric or intestinal mucosa into the blood stream and so into the kidney. On the other hand, we have never seen any sporozoites in the glomerular tuft or capsular space.

The ring forms are not numerous, but they are easily found in heavily infected kidneys. They differ markedly from any stages of the sporoblast cycle and do not appear to be an integral part of it. It is easy to differentiate between a mother sporoblast, for instance, and a ring form seen from the outside, or cut on a tangent, so that the annular appearance is not seen. Moreover, the resulting division or segments of the two forms are apparently very dissimilar. The

sporozoites are slender fusiform bodies, 1 by 4  $\mu$ , with a tiny dot of chromatin; the ring segments are oval or ovoid, 3 by 5 to 7  $\mu$ , with a relatively large mass of chromatin. The ring segments are apparently identical with the small hyaline forms seen free in the lumen of the renal tubules or in the epithelial cells, and there is nothing in the nature of a spore membrane about the dividing ring to prevent their escape into the tubules when the ring completely segments. The final interpretation is, of course, one of conjecture only, but the cycle is strongly suggestive of a sexual phase. The comparatively large number of ring segments or hyaline forms suggests further that they may be the microgametes, and that the ring form may be the microgametocyte. We have not seen any bodies which we could interpret as macrogametes, or any process of fertilization or conjugation, unless Fig. 16 represents this phase.

Smith and Johnson describe in the sporoblast stage of *Klossiella muris* a budding process in which the chromatin occupies the periphery of the budding masses. One might think that the ring form is simply a cross section of some of these buds of the mother sporoblast. But the ring form possesses not only a different type of protoplasm from the mother or daughter sporoblast, but in addition its segments are totally unlike either the daughter sporoblasts on the one hand or the sporozoites on the other.

The asexual or schizogonic cycle is apparently represented by the large extracellular segmenting form seen in Fig. 17. It resembles the glomerular body described by Smith and Johnson and interpreted by them as the schizogonic form. We have not seen it in the glomerular space, but only in the convoluted tubules and its scarcity may be tentatively explained on the assumption that such a stage is present only in early and light infections. Later, apparently, the sporoblast cycle may supersede the schizogony, for this cycle only has been detected in our specimens of heavily infected kidneys. Its extremely large size and the great number of its segments or divisions preclude its belonging to either the sporoblast or ring cycles. Moreover, its situation free in the convoluted tubules is one of the locations where one would expect to find the development of such a stage, if, as we have already suggested, the infecting sporozoites find their way into the kidney by the blood stream. The merozoites or

segments of this large body are similar to the sporozoites, the final divisions of the sporoblast cycle. The conspicuous difference between the two stages of segmentation, aside from the difference in their number, is that the sporozoites are enclosed in a definite spore membrane, while the existence of a membrane surrounding the merozoite is problematical. In the specimens we have seen there is a very indistinct membrane, which, as in Fig. 17, is apparently ruptured, allowing the escape of the merozoites into the renal tubules.

Seidelin found no glomerular bodies as described by Smith and Johnson for *Klossiella muris*, but he considers that some of his tubular forms appear identical with the glomerular forms depicted by them. Unfortunately, Seidelin gives no illustration of this particular tubular form, so that we are unable to compare satisfactorily the two apparently corresponding stages. Seidelin is inclined to the opinion that these tubular forms represent the schizogony. Smith and Johnson think that their glomerular body is the schizogony for *Klossiella muris* and that the tubular forms are stages in the sporoblast cycle.

The pathological changes in the kidney of guinea pigs which we associate with the presence of this parasite are slight but definite and consist of an irregular accumulation of round cells and fibroblasts about some of the glomeruli. There is but slight involvement of the labyrinthine tissue adjacent to the glomeruli and apparently none at all in the lower or inner half of the cortex where the majority of the parasites are found. This may be due to the fact that the infecting sporozoites enter the kidney by way of the glomerular capillaries and that here the most serious injury to the kidney occurs, with a subsequent infiltration of round cells and fibroblasts.

#### SUMMARY.

We have found in the kidneys of twelve supposedly normal guinea pigs, coming from Pennsylvania and New Jersey, a parasite that closely resembles in some of its phases *Klossiella muris*, described by Smith and Johnson, and the renal parasite of two West African guinea pigs, described by Seidelin.

The forms most commonly found by us and described as the sporoblast cycle, are evidently similar to those described by Smith



and Johnson and by Seidelin. There are certain discrepancies of measurement between the parasite described by Seidelin and the one here described, but the most important difference between the two is the different number of sporozoites resulting from a final division of the daughter sporoblasts. Seidelin has found thirty sporozoites; we have found from eight to twelve, while the usual number is eight. Further, we have found a ring form which is unlike any of the stages in either the sporoblast or schizogonic cycle, and which we interpret tentatively as the male element or microgamete. In addition, we have found a tubular form which resembles the glomerular body of *Klossiella muris* and which we think is the schizogonic phase of this parasite.

#### EXPLANATION OF PLATES.

The illustrations are all from untouched microphotographs except Fig. 8, which is a drawing of an actual microscopic field. All the specimens except Fig. 17 are from Zenker fixed tissue.

##### PLATE 63.

FIG. 1. Renal tubules showing a heavy parasitic infection in different stages, the majority being daughter sporoblasts. Hematoxylin and eosin.  $\times 675$ .

FIG. 2. Eight daughter sporoblasts enclosed in an epithelial cell of a convoluted tubule. Hematoxylin and eosin.  $\times 1,000$ .

FIG. 3. Three spores containing seven to nine sporozoites. Note the distinct spore membrane. Hematoxylin and eosin.  $\times 1,000$ .

##### PLATE 64.

FIG. 4. Mother sporoblast enclosed in an epithelial cell. The cell outline is not distinct in the photograph. Giemsa's stain.  $\times 1,000$ .

FIG. 5. Glomerulus containing a single parasite, probably a young mother sporoblast in the capsular space. Hematoxylin and eosin.  $\times 1,000$ .

FIG. 6. Three greatly enlarged epithelial cells, two containing mother sporoblasts, the third in the center containing eleven daughter sporoblasts. Hematoxylin and eosin.  $\times 1,000$ .

##### PLATE 65.

FIG. 7. Two ring forms in adjoining tubules, each enclosed in an enlarged epithelial cell. The ring to the right shows ten divisions. Hematoxylin and eosin.  $\times 1,000$ .

FIG. 8. Two ring forms in distended epithelial cells; the lower form is seen from the outside. Hematoxylin and eosin.  $\times 1,000$ .

## PLATE 66.

FIG. 9. Two ring bodies and several daughter sporoblasts. The ring body on the left is a tangential section through the edge of a hollow sphere. Methylene blue and eosin.  $\times 1,000$ .

FIG. 10. The same field as in Fig. 9, but at a lower level, showing that the so called ring body is a section through a hollow sphere. Methylene blue and eosin.  $\times 1,000$ .

## PLATE 67.

FIG. 11. Two intracellular hyaline forms, the one to the right showing chromatin. Hematoxylin and eosin.  $\times 1,000$ .

FIG. 12. An extracellular spindle-shaped hyaline body showing the chromatin mass at one pole and a clear non-staining area at the opposite pole. One extremity of this hyaline form is in the protoplasm of an epithelial cell. Hematoxylin and eosin.  $\times 1,000$ .

FIG. 13. An intracellular hyaline form in an enlarged epithelial cell. The chromatin has divided into two masses. The parasite is in a vacuole in the host cell. Hematoxylin and eosin.  $\times 1,000$ .

FIG. 14. Two intracellular hyaline forms; the chromatin of the upper parasite has divided into four masses. Giemsa's stain.  $\times 1,000$ .

## PLATE 68.

FIG. 15. Two small intracellular hyaline forms. Giemsa's stain.  $\times 1,000$ .

FIG. 16. Two intracellular hyaline bodies. The larger one shows two elongated chromatin masses. The smaller parasite to the left is at a level which shows no chromatin. Giemsa's stain.  $\times 1,000$ .

FIG. 17. A schizogonic form showing thirty to forty merozoites. This body is free in the tubule and has no definite membrane surrounding it. Taken from a frozen section fixed in 10 per cent formalin. Hematoxylin and eosin.  $\times 1,000$ .

## PLATE 69.

FIG. 18. Irregular cellular infiltrations, especially about the base of some of the glomeruli. Hematoxylin and eosin.  $\times 125$ .

FIG. 19. Three glomeruli showing very slight cellular infiltration about the base. Two glomeruli show no such infiltration. Hematoxylin and eosin.  $\times 210$ .

## PLATE 70.

FIG. 20. The two lower glomeruli show a slight irregular accumulation of round cells and fibroblasts. The upper glomeruli are normal. Hematoxylin and eosin.  $\times 210$ .

FIG. 21. The two glomeruli to the right show a fairly extensive accumulation of round cells and fibroblasts with an irregular extension into the labyrinth. The glomeruli to the left are practically normal. Hematoxylin and eosin.  $\times 210$ .







(Pearce: *Klosiella* Infection of the Guinea Pig.)









(Pearce: *Klosiella* Infection of the Guinea Pig.)



(Pearce: *Klosiella* Infection of the Guinea Pig.)



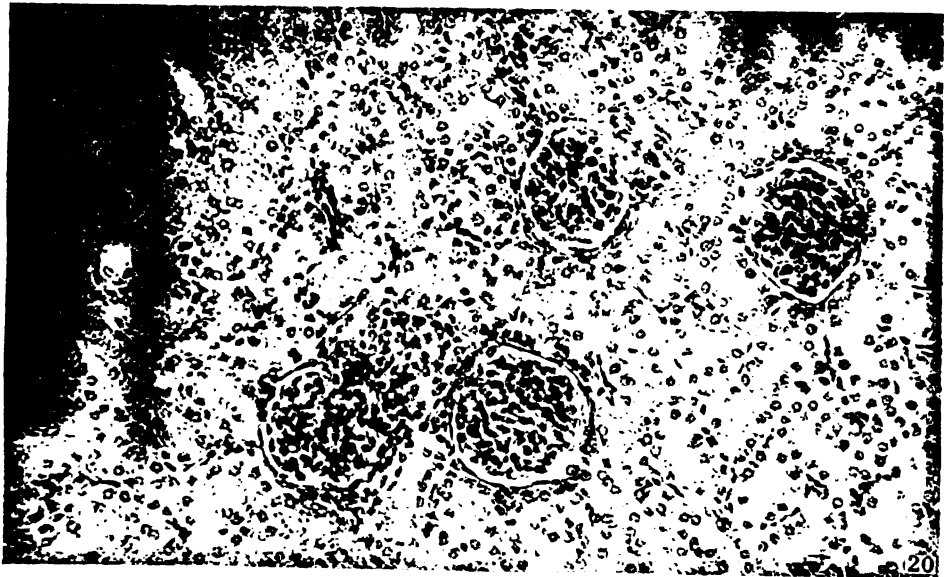
(Pearce: *Klosiella* Infection of the Guinea Pig.)



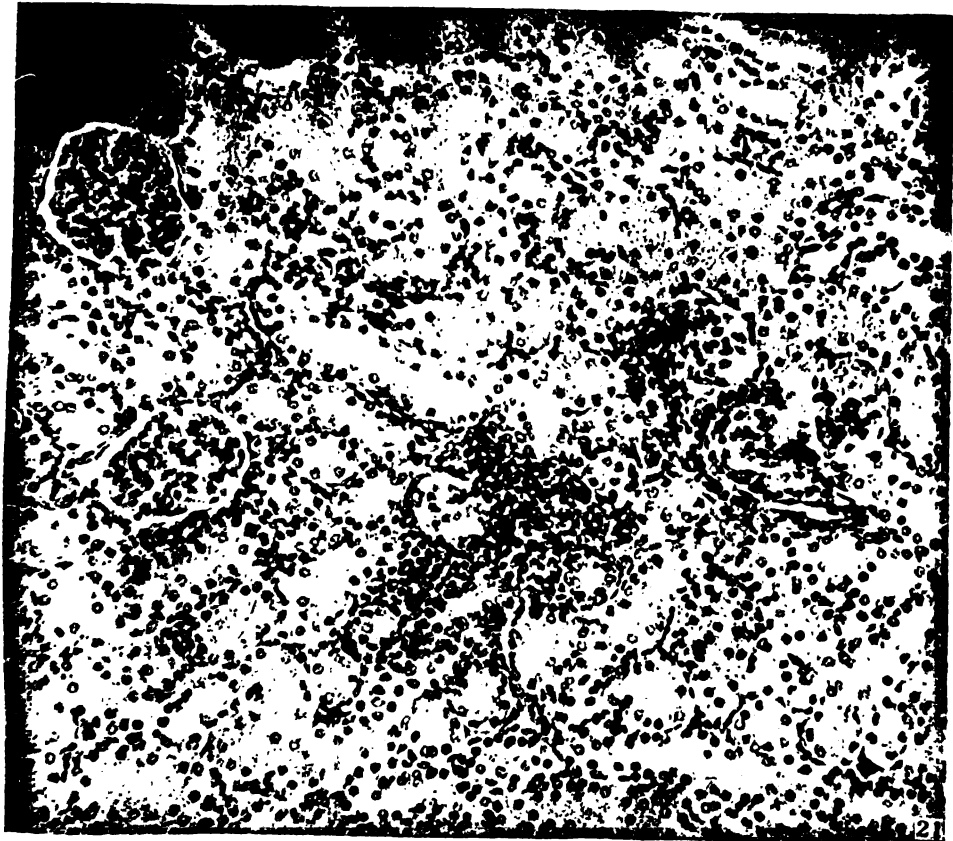








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(Pearce: Klebsiella Infection of the Guinea Pig.)



## CHEMOPATHOLOGICAL STUDIES WITH COMPOUNDS OF ARSENIC.

### IV. THE CHARACTER AND DISTRIBUTION OF RENAL INJURY PRODUCED BY ARSENICALS AS INDICATED BY THE PROCESSES OF REPAIR.

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PLATES 71 TO 76.

(Received for publication, January 31, 1916.)

In previous papers<sup>1</sup> attention was directed to some of the striking differences in the gross and histological changes produced in the kidneys of dogs by lethal doses of certain compounds of arsenic. Upon the basis of these observations it was pointed out that arsenicals could produce a so called tubular as well as a vascular injury of the kidney and that innumerable combinations of these two fundamental forms of tissue injury were obtainable through the use of arsenical compounds of different chemical constitution.

In order to obtain additional information as to the character and location of the specific renal injury produced by these arsenicals, as well as a knowledge of the subsequent processes of organic repair, we have extended our experiments to a study of tissue changes in the kidneys of animals given sublethal doses of these substances.

#### EXPERIMENTAL.

In the following experiments guinea pigs were used, as in our experience the kidneys of these animals are as free from spontaneous lesions as those of any animal available for the purpose. However, it should be mentioned in this connection that in the kidneys of some guinea pigs we have found certain pathological changes that seem to be associ-

<sup>1</sup> Pearce, L., and Brown, W. H., *Jour. Exper. Med.*, 1915, xxii, 517, 525.

acid reveals characteristic changes that differentiate them rather sharply from those of arsenious acid. In the inner half of the cortex and in the boundary zone there is a considerable amount of young fibroblastic (connective) tissue extending for the most part along the medullary rays, but also invading the labyrinth to an appreciable degree. Some of the straight and convoluted tubules in these areas are compressed, but many more are dilated and may contain a granular precipitate (Fig. 2). The epithelium of some of these tubules shows degeneration and is flattened and cuboidal in type. In others it is basophilic and evidently of recent formation. Occasionally mitoses are seen. A few of the tubules in these areas appear as more or less solid cords of recently formed epithelial cells, which stain an intense blue with hematoxylin. The glomeruli in the kidneys of this series are practically normal.

TABLE II.  
*Arsenic Acid.*

Animal No.	Day of experiment and dose of drug in mg. per kilo of body weight.							Doses.	Days survived.	Termination.
	1	2	3	5	6	8	10			
2	2		4		4	10	10	5	13	Killed.
3	8							1	6	"
6	10	10		10				3	7	"

In Guinea Pig 2 (Table II), which received five doses of arsenic acid and survived 13 days, many of the blood vessels in the boundary zone are somewhat thickened. In certain glomeruli some of the capillary walls are apparently thickened also, but this is such an irregular finding and one that may be so variously interpreted that we hesitate to lay much emphasis upon it. The fibroblastic proliferation in the inner cortex and boundary zone is quite characteristic in this animal (Fig. 2). The amount of connective tissue proliferation in Guinea Pig 3 is less than in Guinea Pig 2, but the location and arrangement is the same. In Guinea Pig 6, on the other hand, there is slightly more connective tissue than in Guinea Pig 2, which may be due to the fact that Guinea Pig 6 survived the administration of three comparatively large doses of arsenic acid for 7 days.

In contrasting arsenious and arsenic acid, which resemble each other so closely in the character of the acute renal lesion, it appears that there are distinct differences in their action which are revealed in the process of repair. With arsenious acid, proliferative changes are almost wanting and are practically exclusively confined to the slight increase in nuclei of the glomerular tuft and to slight fibroblastic accumulation about the base of the glomeruli. With arsenic acid, on the other hand, proliferative activity of fibroblasts is appreciably more marked, and is associated with distortion of the tubules especially in the inner half of the cortex and boundary zone and with distinct (although slight) regeneration of tubular epithelium.

*Sodium Cacodylate.*

TABLE III.

*Sodium Cacodylate.*

Animal No.	Day of experiment and dose of drug in mg. per kilo of body weight.				Doses.	Days survived.	Termination.
	1	2	5	6			
2	50	100		1,000	3	7	Died.
3	400		1,000		2	6	"
4	700				1	2	Killed.
5	700		700		2	6	Died.
6	500	500			2	3	"

The kidneys of guinea pigs poisoned with lethal doses of sodium cacodylate belong to the red type and resemble those of arsenious and arsenic acid. In the kidneys of guinea pigs which have survived 2 to 7 days, as is shown in Table III, we find practically the same slight proliferative activity that was observed in the animals which received sublethal doses of arsenious acid. There are occasional small accumulations of fibroblasts and round cells about the glomeruli, along the medullary rays, and in the boundary zone (Fig. 4). In some of the glomerular tufts there is a distinct increase in leukocytes of the endothelial type. There is nothing comparable to the fibroblastic proliferation with consequent distortion of tubules that was comparatively conspicuous in the animals poisoned with arsenic acid. However, in addition to the accumulation of fibroblasts there is more

necrosis of the tubular epithelium than with either arsenious or arsenic acid, and mitoses may be found in both the convoluted and the straight tubules.

There is but little difference, histologically, in the kidneys of the various guinea pigs of this series (Table III). In Guinea Pig 2 there are perhaps more and slightly larger accumulations of fibroblasts. Mitoses are found in the tubular epithelium of all five animals, but they are more numerous in Guinea Pig 6.

There is scarcely any fibroblastic proliferation, therefore, in the animals poisoned with sublethal doses of sodium cacodylate as well as with arsenious acid,—in this respect differing from those poisoned with arsenic acid. But, in addition, there is distinct reparative activity on the part of the tubular epithelium, indicating that this tissue has been injured by the drug, and in this respect differing from guinea pigs poisoned with sublethal doses of both arsenious and arsenic acids, in which the tubular epithelium is but little affected.

#### *Salvarsan.*

We have but one instance in which a sublethal dose of salvarsan was given and the animal allowed to live for more than 24 hours. This guinea pig received 150 mg. of the drug per kilo of body weight and was killed 48 hours after its administration.

The acute salvarsan kidney is a red kidney. In this one example of an early stage in the process of repair the glomeruli are large and rather irregular. The tufts on the whole are not shrunken and there is an accumulation of endothelial leukocytes in some of them. Many of the walls of the tuft capillaries are hyaline and distinctly thickened. About the base of some of the glomeruli there is a collection of fibroblasts and round cells which in some instances extends slightly outward into the labyrinth between the convoluted tubules. In the boundary zone and along the inner portion of the medullary rays are small irregular patches of young fibroblasts (Fig. 5), but there is no distortion of the tubular structures. The tubular epithelium is markedly degenerated but there is no definite epithelial necrosis, and since we have seen no mitoses we may infer that the tubular epithelium was not seriously injured. It should be stated in this

connection, however, that this animal received the dose of salvarsan intraperitoneally and that the compound was absorbed relatively slowly, for when the animal was killed 2 days after injection, the abdominal cavity contained a quantity of unabsorbed drug.

The evidences of repair, seen in the kidneys of guinea pigs after salvarsan injection, are therefore chiefly the changes in the glomeruli and the irregular proliferation of fibroblasts about the glomeruli and in the boundary zone.

### *Neosalvarsan.*

The acute neosalvarsan kidney resembles those of arsenious acid and salvarsan. In Guinea Pig 1, killed on the 2nd day of survival, as shown in Table IV, we find definite signs of a proliferative activity

TABLE IV.

### *Neosalvarsan.*

Animal No.	Day of experiment and dose of drug in mg. per kilo of body weight.		Doses.	Days survived.	Termination.
	1	5			
1	300		1	2	Killed.
2	190	500	2	6	Died.

in the kidney in addition to the acute lesion which is still present. About the base of the glomeruli are small accumulations of fibroblasts and round cells. In addition there is a slight diffuse infiltration of the same character along the medullary rays in both cortex and medulla which in some areas invades the adjacent labyrinthine tissue. The glomeruli themselves are extremely irregular; some are of normal size, while others are markedly shrunken with a contracted tuft filling approximately three-quarters of the capsular space. The tubular epithelium is considerably degenerated and in the inner portion of the cortex there is some individual cell disintegration of the epithelium of the loops of Henle and a slight degree of necrosis. A few mitotic figures are seen.

The changes in Guinea Pig 2 are of the same general character as those in Guinea Pig 1, although of a lesser degree. This may be

due to the much smaller initial dose. The capsules of Bowman and the capillary walls of some of the glomerular tufts are slightly hyaline and thickened. There are small, irregular patches of fibroblasts and round cells about the glomeruli, along the medullary rays, and in the boundary zone. The tubular epithelium is degenerated with occasional slight necrosis. No mitoses are seen.

The changes in the process of repair after neosalvarsan resemble in general those after salvarsan, differing only in the greater irregularity of the glomeruli and the somewhat greater degree of tubular necrosis which is most marked in the inner portion of the cortex. After both salvarsan and neosalvarsan there is a patchy irregular proliferation of fibroblasts in the boundary zone and cortex, but there is no distortion of the tubules as with arsenic acid.

*Arsacetin.*

TABLE V.

*Arsacetin.*

Animal No.	Day of experiment and dose of drug in mg. per kilo of body weight.				Doses.	Days survived.	Termination.
	1	5	13	16			
1	125	250			2	6	Killed.
2	100	200	200	200	4	19	"
3	300				1	2	"

The kidneys of dogs and guinea pigs which have received lethal doses of arsacetin are pale and show a most extensive tubular necrosis with a subordinate vascular injury. In the reaction from a non-fatal dose of this compound there is an exceedingly active and prompt regeneration of the epithelium, especially in the loops of Henle, and to a somewhat less extent in the convoluted tubules and the tubules of the medulla (Figs. 6, 7, and 8). The glomerular capillaries are somewhat dilated and there may be a slight and irregular accumulation of fibroblasts and polyblasts about the base of the glomeruli, along the medullary rays, and in the boundary zone. In Guinea Pig 3 (Table V), killed on the 2nd day, there is already marked activity of the tubular epithelium throughout the cortex and to a less extent in



the medulla. There is practically no vascular alteration and no fibroblastic proliferation in this animal. Guinea Pig 1 was killed on the 6th day after having received two doses of arsacetin. The kidney sections of this animal show a moderate regeneration of the tubular epithelium, almost exclusively confined to the loops of Henle and the medullary tubules. About the base of some of the glomeruli there is a very slight degree of fibroblastic proliferation. Four moderate sized doses of arsacetin were given to Guinea Pig 2 in 16 days, and the animal was killed on the 19th day (Table V). In this animal the regenerative activity of the tubular epithelium has reached a remarkable degree. Mitotic figures are found in practically every microscopic field (4 mm. Zeiss objective; No. 4 ocular) and they are particularly numerous in the straight tubules of the cortex and medulla (Figs. 6, 7, and 8). Many of the tubules along the medullary rays show entirely new epithelium and some of these tubules are almost solid masses of new cells. Degeneration with some necrosis and desquamation of cells is still present in some of the convoluted tubules and limbs of Henle and there are many hyaline casts in the boundary zone and medulla. In the boundary zone, along the medullary rays, and about the base of some of the glomeruli there is a rather diffuse proliferation of fibroblasts and infiltration of polyblasts which irregularly invade the labyrinth in some areas. Several of the renal tubules of this animal contain the parasite which we have referred to and which must be taken into consideration in interpreting the proliferative changes about the glomeruli.

#### *Atoxyl.*

The acute lesion in the kidneys of dogs and guinea pigs poisoned with lethal amounts of atoxyl is predominantly tubular, but the vascular changes are by no means inconspicuous. In guinea pigs which have received sublethal doses of atoxyl, there is a rapid attempt at regeneration of the tubular epithelium which is shown in both animals of this series (Table VI). Many of the straight tubules are dilated and lined with low cuboidal basophilic staining cells (Fig. 9), and there are numerous mitoses in both cortical and medullary epithelium (Figs. 9, 10, and 11). There is a well marked leukocytic exudate

in the interstitial tissue as well as in some of the tubules of Guinea Pig 3 (Fig. 11); it is less prominent in Guinea Pig 1. In addition to the marked epithelial necrosis with regeneration and the cellular exudate caused by the injection of atoxyl, there is a definite fibroblastic proliferation along the medullary rays, in the boundary zone, and in the upper medulla (Figs. 11 and 12).

The territorial distribution of fibroblastic proliferation in Guinea Pig 3 corresponds with that in Guinea Pig 1. The process, however, is distinctly more pronounced, as might be expected, the animal having received three doses of atoxyl in 8 days. The fibroblasts are somewhat diffusely distributed throughout the lower edge of the cortex, and along the boundary zone and upper medulla. Some of the tubules in the area of fibroblastic proliferation are compressed,

TABLE VI.

*Atoxyl.*

Animal No.	Day of experiment and dose of drug in mg. per kilo of body weight.			Doses.	Days survived.	Termination.
	1	2	5			
1	100			1	3	Killed.
3	50	50	50	3	8	"

while others are slightly dilated (Figs. 9 and 12). The glomeruli are irregular in both animals. Some are quite large, others are shrunken. Many of the tuft capillaries are dilated and the walls moderately thickened. About the base of the glomeruli are slight fibroblastic accumulations which in a few areas invade the adjacent labyrinth.

The process of repair in the kidneys of guinea pigs poisoned with sublethal doses of atoxyl resembles that of arsacetin in the prompt and marked regeneration of tubular epithelium. Moreover, there is a definite proliferation of fibroblasts which recalls the changes caused by arsenic acid, and in addition there is a well marked exudation of polymorphonuclear leukocytes into the interstitial tissue and tubules.

*Arsenophenylglycine.*

Arsenophenylglycine acts somewhat differently in guinea pigs and in dogs. With lethal amounts of the drug the kidneys of dogs and guinea pigs are both pale, with a predominant tubular necrosis and a relatively subordinate vascular injury. In the guinea pig, however, after a comparable sublethal dose of the compound there is but little tubular necrosis and but slight vascular injury. Consequently, after such a dose of arsenophenylglycine the process of repair in the kidney consists only in recovery from a parenchymatous and fatty degeneration of the tubular epithelium with now and then a mitotic figure.

TABLE VII.

*Arsenophenylglycine.*

Animal No.	Day of experiment and dose of drug in mg. per kilo of body weight.		Doses.	Days survived.	Termination.
	1	5			
1	250		1	2	Killed.
2	250		1	5	"
3	100	50	2	6	Died.
4	400		1	3	Killed.
5	300	300	2	6	Died.
6	500		1	4	Killed.

Such a course of events is followed after a single or a repeated sublethal dose (Table VII). In Guinea Pig 1, for instance, killed on the 2nd day, degeneration of tubular epithelium is marked, but there is practically no cell necrosis, and no mitotic figures are seen. Guinea Pig 2 received the same amount of arsenophenylglycine as Guinea Pig 1, but was not killed until the 5th day. The epithelial changes in the two animals are almost identical. In Guinea Pig 2 there is an increase of tuft nuclei, mostly of the polymorphonuclear variety, and about the base of some of the glomeruli are slight accumulations of fibroblasts. Guinea Pig 3 received two small doses of the compound, and the alterations here are much the same as in the first two animals. The leukocytic cells infiltrating the glomerular

tuft are mostly eosinophilic; there is a very irregular thickening of the wall of the glomerular capillaries and an extremely irregular and slight distribution of round cells and fibroblasts about the base of some of the glomeruli, along the straight vessels, and in the labyrinth. The tubular epithelium is swollen and granular and there is some necrosis of a disintegrative character in the epithelium of the outer cortex. An occasional mitotic figure is seen.

The acute changes in the kidneys of guinea pigs after sublethal doses of arsenophenyglycine consist almost entirely of parenchymatous and fatty degeneration of the tubular epithelium with but little cell necrosis, differing in this respect from a similar sublethal injury in the kidneys of dogs. The processes of repair after such an injury are, therefore, comparatively simple and do not involve any appreciable degree of cell regeneration. There is only a slight and very irregular interstitial fibroblastic proliferation.

#### DISCUSSION.

A study of the processes of repair in the kidneys of guinea pigs poisoned with sublethal doses of certain arsenical compounds furnishes additional information as to the character and location of the acute injury. The idea, which we have previously suggested, namely, that all arsenicals do not produce a purely vascular type of renal injury, is further substantiated by this series of experiments in which the regeneration of tubular epithelium plays a relatively conspicuous part. The participation of the epithelial tissue, however, is by no means the dominant feature in the recovery after sublethal doses of all arsenical compounds, but only after certain particular ones. In others, the acute injury is mainly vascular, and the reaction of the epithelial structures is but slight. Further, the interstitial proliferation of fibroblasts which occurs in a marked degree after injections of various arsenicals may be especially pronounced in the repair following a drug which, in lethal doses, causes but little vascular disturbance, as, for instance, arsacetin. Here, the initial and dominant injury is epithelial; there is only slight demonstrable alteration of the vascular structures. In the process of recovery, however, after the administration of sublethal doses of arsacetin, there may be quite

a marked proliferation of young fibroblasts diffusely distributed (Table V, Guinea Pig 2, and Fig. 8). This is also seen in the repair of the kidney following injections of atoxyl, although here the picture is more complicated, for atoxyl affects the vascular as well as the epithelial tissue of the kidney. The distribution of the proliferating fibroblasts after a more purely vascular injury such as that produced by arsenious or arsenic acid is confined more sharply to the boundary zone, with radiations along the medullary rays to the glomeruli. On the other hand, after arsacetin, the fibroblastic proliferation is more diffusely distributed throughout the cortex, although it may be more numerous in the boundary zone and along the medullary rays.

Taking all these observations into consideration, therefore, it would seem that a toxic agent like arsenic, may, in one or another of its various combinations, injure the vascular, epithelial, or interstitial (connective) tissue of the kidney, if we may judge of such an initial injury by the subsequent processes of repair. The relative distribution and extent of the initial injury may be difficult to determine until one studies various stages of the recovery of the kidney, in which the injured tissues are regenerating. This is particularly true in the case of injury of the connective tissue of the kidney.

After a sublethal dose of arsenious acid which produces an almost pure type of vascular injury in the kidneys, the return to normal is very rapid and there is only a slight fibroblastic proliferation about the base of the glomeruli to indicate a previous injury. Injections of sublethal amounts of salvarsan also cause a proliferation of fibroblasts of a more interstitial and rather patchy character, somewhat greater in extent and amount than with arsenious acid. On the other hand, with arsenic acid, there is a relatively large amount of fibroblastic proliferation, especially in the boundary zone, resulting in the compression or dilatation of some of the tubules, and in addition a slight although definite regeneration of tubular epithelium. Sodium cacodylate in sublethal amounts causes essentially the same slight fibroblastic changes seen after arsenious acid, but a well marked regeneration of tubular epithelium as well. With all these four compounds, arsenious and arsenic acid, salvarsan, and sodium cacodylate, there are slight but fairly regular glomerular changes, consisting of

a slightly swollen tuft and an increase in the tuft nuclei. With neosalvarsan, however, the glomeruli are extremely irregular, some being very large, others contracted and shrunken. There is a slight interstitial fibroblastic proliferation, comparable to that of salvarsan and a slight regeneration of tubular epithelium. After sublethal injections of atoxyl, there is very marked regenerative activity of the tubular epithelium, a leukocytic exudate, and a definite and rather diffuse interstitial proliferation of fibroblasts with dilatation or compression of adjacent tubules. Following a sublethal injection of arsacetin, there is a conspicuous and prompt regeneration of tubular epithelium and a relatively diffuse interstitial proliferation of fibroblasts. Unfortunately, we must omit the experiments of arsenophenylglycine on the guinea pig in this consideration, for the reasons stated above.

It would seem, therefore, that arsenicals which produce an injury that is primarily vascular may lead to only a slight subsequent proliferation of tissue (arsenious acid). However, other arsenicals which produce an acute injury that is vascular, may lead to distinct interstitial proliferation (arsenic acid, salvarsan, and neosalvarsan). Moreover, an arsenical that produces an acute injury that is primarily vascular may also produce injury of the tubular epithelium which in the stages of repair dominates the picture to the exclusion of any extensive interstitial proliferation (sodium cacodylate).

On the other hand, arsenicals that produce primarily an injury of tubular epithelium cause a marked subsequent regeneration of this tissue, and may cause in addition an interstitial fibroblastic proliferation (arsacetin). But an arsenical compound that produces a marked vascular injury in addition to the picture of tubular necrosis may lead to a relatively marked interstitial proliferation as well as the extensive epithelial regeneration (atoxyl).

In connection with the fibroblastic proliferation described in the processes of repair in these kidneys, we must refer to the presence of a parasite in the kidneys of some guinea pigs.<sup>2</sup> This parasite is only occasionally found in guinea pigs, but we believe that it may cause irregular accumulations of fibroblasts and round cells about the base of some of the glomeruli and in the neighboring labyrinthine tissue. These accumulations resemble those seen after sublethal injection

of arsenious acid, but they are much more irregular. Therefore, we wish to be particularly cautious in a final interpretation of our results and to take into consideration the possibility that some of the fibroblastic proliferation we have seen in the kidneys of guinea pigs after sublethal injections of these arsenicals may be due in part to this renal parasite.

We have shown that after acute lethal injury with various arsenicals, two types of kidneys could be distinguished, both grossly and histologically, namely, the red and the pale. Further, in the reaction after a sublethal injury inflicted with these compounds these two main types of kidney alteration may still be differentiated by the processes of repair. The administration of those compounds which produce a predominantly pale kidney is followed by a most prompt and pronounced regeneration of tubular epithelium with a varying degree of diffuse fibroblastic proliferation. The compounds that produce a predominantly red kidney are followed by proliferation of fibroblasts with but slight regeneration of the tubular epithelium. The distribution of the fibroblasts in the reparative stages apparently corresponds in some degree to the initial injury. After a more or less predominant vascular injury the fibroblasts are usually found about the glomeruli, along the medullary rays, and in the boundary zone. With other compounds that show little evidence of an acute vascular injury, the connective tissue injury may be more pronounced, and in recovery from such an injury the fibroblasts are diffusely distributed throughout the cortex and medulla in a typical interstitial manner. But, just as there are innumerable combinations of vascular and tubular and probably connective tissue injury in the acute arsenic kidneys, so there are innumerable combinations of the different tissue elements in the processes of repair. Hence, we suggest that the character and distribution of renal injury produced by arsenical compounds as indicated by the processes of repair are bound up in the chemical constitution of these compounds. Further, that each particular compound as far as the kidney is concerned, acts as a more or less specific toxic agent, as shown by the character and distribution of the renal lesions.

## SUMMARY.

1. The processes of repair in the kidneys of guinea pigs after sub-lethal doses of certain arsenical compounds indicate that all arsenicals do not produce a purely vascular type of renal injury.

2. While some arsenicals produce a predominantly vascular injury and others produce a predominantly tubular injury, both these tissue elements are undoubtedly always affected, although in varying proportion. In addition, the interstitial connective tissue is probably always affected. The diffuse proliferation of this tissue may be relatively conspicuous in the processes of repair after arsenicals that cause but slight vascular injury.

3. All red kidneys do not necessarily show identical pictures during the processes of repair; the same is true of pale kidneys.

4. The mode of action of an arsenical compound as a renal toxic agent is bound up with the chemical constitution of the compound.

## EXPLANATION OF PLATES.

The illustrations are all from untouched microphotographs.

## PLATE 71.

FIG. 1. Arsenious acid. Guinea Pig 2. Section from the outer cortex. There is a regular and definite although slight accumulation of round cells and fibroblasts about the glomeruli. The nuclei of the glomerular tuft show a slight increase. The tubular epithelium is normal.  $\times 210$ .

FIG. 2. Arsenic acid. Guinea Pig 2. Section from the inner cortex. There is a slight interstitial proliferation of connective tissue, and the renal tubules in this area are somewhat distorted, some being dilated and others compressed. The tubular epithelium and glomeruli are practically normal.  $\times 210$ .

## PLATE 72.

FIG. 3. Arsenic acid. Guinea Pig 6. Section from the boundary zone. There is a small but conspicuous proliferation of connective tissue with consequent dilatation and compression of the renal tubules in this area. The renal epithelium is swollen and shows parenchymatous degeneration.  $\times 210$ .

FIG. 4. Sodium cacodylate. Guinea Pig 2. Section from the outer cortex. The glomerular tuft is slightly swollen and shows a slight increase of tuft nuclei. There is a definite and regular although slight infiltration of round cells and fibroblasts around the base of the glomeruli. The renal epithelium is slightly swollen and degenerated.  $\times 210$ .





























## PLATE 73.

FIG. 5. Salvarsan. Guinea Pig 1. Section from the boundary zone. There is an irregular patchy proliferation of connective tissue. The renal epithelium is practically normal.  $\times 210$ .

FIG. 6. Arsacetin. Guinea Pig 2. Section from the outer cortex. There is a marked degeneration and regeneration of the tubular epithelium with numerous mitotic figures. There is a granular precipitate in the lumen of the tubules which appear slightly dilated. The glomeruli are normal in appearance. There is a slight diffuse proliferation of fibroblasts.  $\times 210$ .

## PLATE 74.

FIG. 7. Arsacetin. Guinea Pig 3. Section from the midcortex. There is a moderate degeneration and marked regeneration of the tubular epithelium with numerous mitotic figures. There is a granular precipitate in the lumen of the tubules.  $\times 210$ .

FIG. 8. Arsacetin. Guinea Pig 2. Section from the upper medulla. There is marked regeneration of the tubular epithelium with numerous mitotic figures. There is a slight but definite interstitial proliferation of fibroblasts.  $\times 210$ .

## PLATE 75.

FIG. 9. Atoxyl. Guinea Pig 3. Section from the inner cortex. There is a conspicuous dilatation of some of the tubules with a granular precipitate in the lumen. The tubular epithelium shows regeneration with numerous mitotic figures. The glomeruli appear normal.  $\times 210$ .

FIG. 10. Atoxyl. Guinea Pig 1. Section from the outer cortex. There are two adjacent mitotic figures in the epithelium of a convoluted tubule.  $\times 1,470$ .

## PLATE 76.

FIG. 11. Atoxyl. Guinea Pig 3. Section from the boundary zone. There is a conspicuous leukocytic infiltration in the tubules and interstitial tissue. The tubular epithelium shows numerous mitotic figures. There is some interstitial proliferation of the connective tissue.  $\times 378$

FIG. 12. Atoxyl. Guinea Pig 3. Section from the boundary zone. There is a diffuse interstitial proliferation of connective tissue. The tubular epithelium shows several mitotic figures.  $\times 378$ .



## EXPERIMENTS WITH POLIOMYELITIS IN THE RABBIT.

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PLATES 77 to 80.

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Inoculation of the common laboratory animals with the virus of poliomyelitis has met with so little success that the disease has been generally regarded as exclusively limited to man and the monkey. Attempts have been made to transmit the virus to many animals, but of all the animals tested, positive results have thus far been recorded only for monkeys, rabbits, and, perhaps, guinea pigs.

Krause and Meinicke<sup>1</sup> reported the passage of a strain of virus obtained from a human case through seven generations in rabbits. Lentz and Huntemüller<sup>2</sup> report having successfully transferred the virus from rabbit to rabbit by various methods of inoculation. They found the alterations in the brain and spinal cord to be slight as compared with those in monkeys. On the other hand, Römer and Joseph,<sup>3</sup> Landsteiner and Levaditi,<sup>4</sup> Leiner and von Wiesner,<sup>5</sup> and Flexner and Lewis<sup>6</sup> all failed to transfer the disease to rabbits. The most striking results, perhaps, have been reported by Marks,<sup>7</sup> who carried a strain of poliomyelitic virus derived from a *rhesus* monkey through seven generations of young rabbits varying in weight from 350 to 550 gm. The animals that succumbed developed no paralysis, but died in convulsions. No lesions definitely characteristic of poliomyelitis could be found on microscopic examination. Marks says: "The disease thus produced in rabbits cannot be recognized as poliomyelitis," although he concludes that filtrates of the nervous tissues of monkeys dying from experi-

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<sup>1</sup> Krause, P., and Meinicke, E., *Deutsch. med. Wchnschr.*, 1909, xxxv, 1825.

<sup>2</sup> Lentz and Huntemüller, *Ztschr. f. Hyg. u. Infektionskrankh.*, 1910, lxvi, 481.

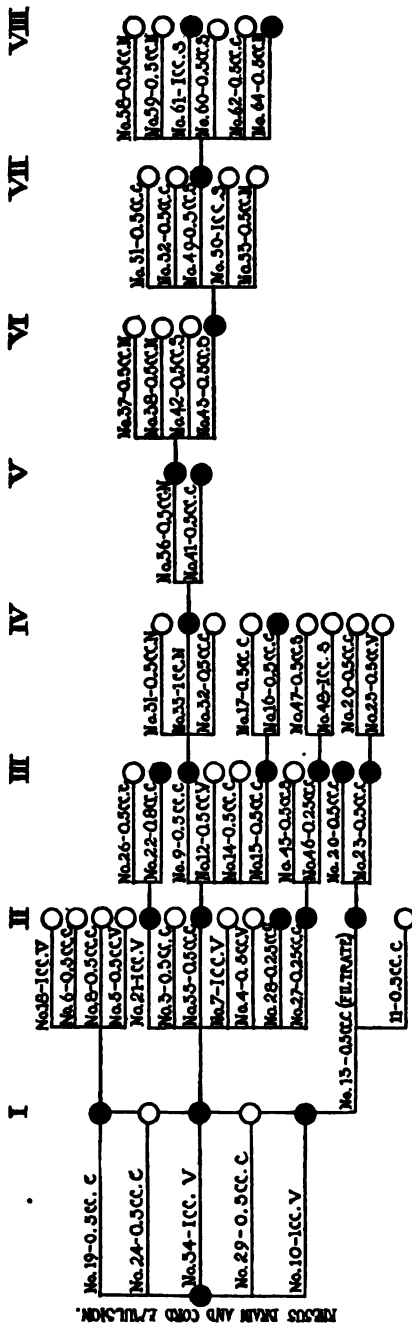
<sup>3</sup> Römer, P. H., and Joseph, K., *München. med. Wchnschr.*, 1910, lvii, 2685.

<sup>4</sup> Landsteiner, K., and Levaditi, C., *Compt. rend. Soc. de biol.*, 1909, lxvii, 787.

<sup>5</sup> Leiner, C., and von Wiesner, R., *Wien. klin. Wchnschr.*, 1909, xxii, 1698.

<sup>6</sup> Flexner, S., and Lewis, P. A., *Jour. Exper. Med.*, 1910, xii, 227.

<sup>7</sup> Marks, H. K., *Jour. Exper. Med.*, 1911, xiv, 116.



TEXT-FIG. 1. Chart showing the progress of the virus through eight generations of rabbits. C, intracerebral inoculation; V, intravenous inoculation; N, nasal insufflation; S, injection into sciatic nerve sheath.



mental poliomyelitis are not wholly innocuous to young rabbits. Marks further states that "not all strains of the virus can be transmitted successfully to even a small fraction of individuals of all varieties of domesticated rabbits." This fact, which we have corroborated, may explain the negative results of other investigators.

Römer and Joseph<sup>8</sup> have observed that guinea pigs in the laboratory occasionally die of a paralytic disease. They were unable to transfer poliomyelitic virus from monkeys to guinea pigs, although Römer<sup>8</sup> found that the spontaneous paralysis was due to a filterable virus. Neustaedter<sup>9</sup> claims to have carried a strain from a guinea pig presumably infected through contact with a monkey into one other guinea pig and back again to a monkey.

In the course of our experiments we have inoculated a few guinea pigs. The lesions in those that succumbed were somewhat similar to the lesions seen in the rabbits. This part of our work is not sufficiently advanced to warrant conclusions. Moreover, we have no criterion by which to establish what is and what is not poliomyelitis, except by reproducing the disease in monkeys. It is, therefore, hazardous to affirm or deny the identity with poliomyelitis of these aberrant conditions in other animal species.

The difficulty of obtaining monkeys on account of the war led us to consider other animals that might be susceptible and therefore suitable for experimental purposes. We selected the rabbit because successful results had previously been obtained with these rodents and because rabbits offered a good chance to compare the action of poliomyelitic with rabic virus. We accordingly inoculated rabbits with poliomyelitic virus from a *rhesus* monkey infected by intracranial inoculation with a strain sent us from The Rockefeller Institute for Medical Research by Dr. Harold L. Amoss. We have obtained positive results in young rabbits and have succeeded in transferring the virus from rabbit to rabbit through eight successive generations.

Intracranial inoculations have been used for the most part, although infection has taken place through intravenous injection, also through injection into the sciatic nerve sheath, and even after introduction of the virus into the anterior nares, upon the uninjured nasal mucosa. A few intraperitoneal injections were tried, but with negative results.

<sup>8</sup> Römer, P. H., *Ergebn. d. inn. Med. u. Kinderh.*, 1912, viii, 1.

<sup>9</sup> Neustaedter, M., *Jour. Am. Med. Assn.*, 1913, ix, 982.

All inoculations are by no means successful. Over one-half fail; we obtained positive results in twenty-two rabbits out of a total of fifty-four inoculated in various ways. Of five rabbits inoculated with the virus from the *rhesus* monkey two have failed to show symptoms, although kept under observation for 5 months.

The age incidence of poliomyelitis in man is indicated by the name "infantile paralysis." In our experimental work we obtained positive results in young rabbits only. We failed to infect three full grown rabbits with *rhesus* virus, although this same virus caught in three out of five young rabbits. Furthermore, rabbit virus from the second and third generations was transferred to rabbits 8 weeks old with negative results. Thereafter we used only young animals under 6 weeks old in our experiments. There seems to be a parallel between rabbits and man as far as susceptibility of the young is concerned. These facts seem to furnish a striking example of natural immunity acquired during the period of adolescence.

The incubation period in our observations of twenty-two rabbits has been variable, the shortest being 2 days, the longest, 41. In this respect our experience is similar to that of Lentz and Huntmüller,<sup>2</sup> who found the incubation period to be of uncertain length, sometimes as long as 2 months, but usually between 7 and 11 days. The average period of incubation in our observations was 12 days. Curiously enough, the shortest period, 2 days, as well as the longest, 41 days, was after intracranial injections.

The virus so far has given no evidence of increasing adaptation to rabbits, or of becoming fixed to any degree, the number of failures being as great, and the period of incubation as variable, in the eighth generation as in the first.

The symptoms also vary. They can, however, be divided roughly into two classes: (1) A type which we designate the progressive type, in which the rabbit first becomes inactive, loses weight, and appears weak. This is followed by partial or complete paralysis of one or more of the extremities (Fig. 1), which usually progresses until death. This corresponds somewhat to the syndrome most commonly seen in experimental poliomyelitis in monkeys. The duration of the disease varies from 1 or 2 days to 1 week. The paralysis is usually flaccid, but occasionally it is spastic. Sometimes the paralysis be-

gins locally. As a rule, it is easy to determine that the paralysis is a true palsy and not simply "weakness." (2) The other group of symptoms, which we designate the fulminating type, is more explosive in character, develops suddenly, and terminates in a very short time; it never extends over more than 2 days and usually lasts only a few hours. The overshadowing symptoms are extreme weakness and marked dyspnea. As an illustration of the rapid course of the symptoms of the fulminating type the case of Rabbit 21 is cited. This rabbit was inoculated intravenously on Aug. 18 with 1 cc. of a rich emulsion of virus from a first generation rabbit (No. 34). On Sept. 12, 25 days later, it still appeared to be normal. In about an hour after this observation it was found by one of us lying flat on its side, breathing in slow, labored gasps. There was great general weakness, the rabbit being unable to raise its head, but no paralysis of the extremities was demonstrable. Within a few minutes the animal was dead, the whole syndrome lasting less than an hour. This is an extreme instance of the fulminating type, and is of special interest since it follows a long period of incubation. We have seen combinations of the two types with symptoms resembling Landry's paralysis.

Most of the animals in which the symptoms lasted over 2 or 3 days lost weight rapidly. In some cases the loss of weight was the first observed symptom.

That the rabbit virus is filterable is shown by the protocol of Rabbit 13 of the second generation. This rabbit was inoculated intracranially with 0.5 cc. of a Berkefeld filtrate of brain and cord emulsion from Rabbit 10. Six days later the hind legs became paralyzed, and the animal died 2 days after onset with symptoms of the fulminating type. The unfiltered virus from Rabbit 13 proved infectious for two other rabbits (Nos. 20 and 23).

We have inoculated a monkey with virus from Rabbit 49 of the seventh generation. The monkey died after an incubation period of 4 weeks, with symptoms somewhat resembling the fulminating type seen in the rabbits. The gross pathological findings were congestion of the pia, especially in the region of the medulla, and hyperemia and hemorrhage of the gray matter of the medulla and cord. The microscopic lesions were similar to those seen in the rabbits and not typi-

cal of experimental poliomyelitis in monkeys. The monkey was a South American species which we have since found to be highly resistant to poliomyelitic virus derived from *rhesus* monkeys. This interesting observation is being made the subject of further study.

Flexner found that *Capucinus* monkeys were resistant, although *Cebus*, another South American species, were susceptible, but less so than Old World monkeys. He therefore concludes that the *Platyrrhines* are less susceptible than the *Catarrhines*.<sup>10</sup>

The intracranial inoculations were done by making a small incision in the skin, near the midline, drawing this to one side, and introducing the needle of the syringe directly through the frontal bone, which is thin and soft in young rabbits. The virus is then injected slowly into the region of the lateral ventricle. On withdrawing the needle, the skin slides back and acts as a valve to cover the small opening in the bone.

The emulsion of virus in all cases was made by grinding in a mortar, portions of the brain and cord with salt solution, and filtering through several layers of sterile gauze.

In Table I are briefly summarized the results of the rabbits which succumbed to intracranial inoculation.

Fourteen rabbits inoculated intracranially failed to show symptoms.

The intravenous inoculations were made into the posterior auricular vein with an emulsion of the central nervous matter prepared as above described.

Table II contains a condensed summary of the rabbits which succumbed to intravenous inoculations.

Six rabbits inoculated intravenously failed to show symptoms.

In order to inject the virus into the sciatic nerve sheath, a short incision was made in the skin above the nerve, the tissues were dissected sufficiently to render the nerve visible, and the needle was then inserted into the sheath in the central direction. The emulsion was prepared as above.

Table III gives a condensed summary of the rabbits which succumbed as a result of injection into the sciatic nerve sheath.

Five rabbits inoculated into the sciatic nerve sheath failed to develop symptoms.

<sup>10</sup> Flexner, S., *Jour. Am. Med. Assn.*, 1910, lv, 1105.

TABLE I.

*Intracranial Inoculation.*

No. of rabbit.	Amount of emulsion.	Rabbit generation.	Period of incubation.	Type of symptoms.*
	cc.		days	
19	0.5	I	19	P.
35	0.5	II	5	P.
27	0.25	II	31	F.
28	0.25	II	34	F.
13	0.5	II	6	F.
20	0.5	III	41	F.
23	0.5	III	9	P.
22	0.8	III	10	P.
46	0.25	III	10	Convulsions.
15	0.5	III	2	F.
9	0.5	III	14	P.
16	0.5	IV	13	P.
41	0.5	V	12	P.

\* In this and the following tables, the letter P indicates symptoms of the progressive type; F, symptoms of the fulminating type.

TABLE II.

*Intravenous Inoculation.*

No. of rabbit.	Amount of emulsion.	Rabbit generation.	Period of incubation.	Type of symptoms.
	cc.		days	
34	1.0	I	18	F.
10	1.0	I	3	F.
21	1.0	II	25	F.

TABLE III.

*Inoculation into the Sciatic Nerve Sheath.*

No. of rabbit.	Amount of emulsion.	Rabbit generation.	Period of incubation.	Type of symptoms.
	cc.		days	
43	0.5	VI	8	P.
49	0.5	VII	6	F.
61	1.0	VIII	20	F.

In order to introduce the virus into the nose, the following procedure was adopted. The rabbit was lightly etherized, held on its back, and the emulsion of the virus then dropped into the anterior nares from an ordinary medicine dropper, care being taken not to injure the mucosa. There was usually a little sneezing immediately after the fluid was introduced.

Table IV is a condensed summary of the three rabbits which succumbed as a result of intranasal insufflation.

TABLE IV.

*Intranasal Insufflation.*

No. of rabbit.	Amount of emulsion.	Rabbit generation.	Period of incubation.	Type of symptoms.
	cc.		days	
33	0.5	IV	2	F.
36	0.5	V	2	F.
64	0.25	VIII	2	F.

Six rabbits inoculated by intranasal insufflation failed to develop symptoms.

It will be seen that, judging from the limited data at hand, this is a particularly virulent way of infecting rabbits with the virus. The period of incubation is short—only 2 days—and the symptoms in all three cases were of the fulminating type. These represent only three out of nine rabbits tested by introducing the virus into the nose. The other six failed to take. Particular attention was paid to the lungs of the rabbits in this group at autopsy. There was no evidence of pneumonia, or even of congestion of the lungs. On microscopical examination it was found that the medulla was markedly congested, whereas the cord was only slightly affected.

The gross lesions consist of injection of the vessels of the pia, hyperemia of the gray matter of the medulla and cord, and more or less marked edema throughout the brain and cord. The microscopic lesions are distributed rather uniformly throughout the gray matter of the cord and medulla in the progressive type. In the fulminating group (those exhibiting symptoms of respiratory failure without

paralysis of the skeletal muscles), the lesions are more marked in the medulla.

The microscopic lesions consist of capillary congestion, focal hemorrhages into the gray matter, degeneration of the large motor cells, and infiltration with cells of uncertain origin (Figs. 2, 3, 4, 5, 6, 7, and 8). These cells for the most part seem to be proliferated glia cells, and therefore appear to be different from the infiltrating lymphocytes of the lesions of poliomyelitis in man. The congestion of the capillaries and small arterioles is conspicuous. The infiltrating cells when stained with eosin and methylene blue have large, vesicular nuclei with a number of conspicuous chromatin granules. The cytoplasm is scanty and homogeneous. They are scattered throughout the gray matter, and are also grouped in satellite arrangement around the nerve cells, but the perivascular infiltration, so typical of the lesions of poliomyelitis in man and the monkey, is absent in the rabbit. Punctate hemorrhages are numerous, sometimes every vessel in a field being ruptured. The nerve cells show all stages of degeneration. Chromatolysis is common and satellitosis is an almost constant feature. A moderate degree of round cell infiltration is occasionally seen in the meninges. The accompanying plates illustrate the different features of the lesions. The complete picture gives the impression of a severe intoxication of the gray matter of the cord and medulla.

The following are the protocols in brief of the rabbits that succumbed, arranged chronologically, as in Text-fig. 1.

*Rabbit 34.*—Age 6 wks. Weight 660 gm. 1st generation. 1 cc. of *rhesus* virus intravenously. Incubation period 18 days. Died in the night with no observed symptoms.

*Rabbit 19.*—Age 5 wks. Weight 600 gm. 1st generation. 0.5 cc. of *rhesus* virus intracranially. Incubation period 19 days. Death in 4 days. Paralysis of front legs with respiratory symptoms.

*Rabbit 10.*—Age 4 wks. Weight 550 gm. 1st generation. 1 cc. of *rhesus* virus intravenously. Incubation period 3 days. Died in the night with no observed symptoms.

*Rabbit 21.*—Age 5 wks. Weight 600 gm. 2nd generation. 1 cc. of virus of Rabbit 34 intravenously. Incubation period 25 days. Death in 1 hour. Symptoms very explosive in character; complete prostration and marked dyspnea.

*Rabbit 35.*—Age 6 wks. Weight 730 gm. 2nd generation. 0.5 cc. of virus of Rabbit 34 intracranially. Incubation period 5 days. Hind legs became

paralyzed. Paralysis remained stationary for 5 days, then showed tendency to improvement. Chloroformed.

*Rabbit 27.*—Age 3 wks. Weight 290 gm. 2nd generation. 0.25 cc. of virus of Rabbit 34 intracranially. Incubation period 31 days. Death in 1 day. Paralysis and dyspnea.

*Rabbit 28.*—Age 3 wks. Weight 255 gm. 2nd generation. 0.25 cc. of virus of Rabbit 34 intracranially. Incubation period 34 days. Death in 1 day. Weakness and dyspnea.

*Rabbit 13.*—Age 4 wks. Weight 540 gm. 2nd generation. 0.5 cc. of Berkeley filtrate of emulsion of virus of Rabbit 10 intracranially. Incubation period 6 days. Death in 2 days. Paralysis of hind legs and respiratory distress.

*Rabbit 20.*—Age 5 wks. Weight 645 gm. 3rd generation. 0.5 cc. of virus of Rabbit 13 intracranially. Incubation period 41 days. Death in 1 day. Explosive symptoms; prostration and marked dyspnea.

*Rabbit 23.*—Age 4 wks. Weight 410 gm. 3rd generation. 0.5 cc. of virus of Rabbit 13 intracranially. Incubation period 9 days. Death in 5 days. General weakness and dyspnea.

*Rabbit 22.*—Age 5 wks. Weight 620 gm. 3rd generation. 0.8 cc. of virus of Rabbit 21 intracranially. Incubation period 10 days. Death in 7 days. Paralysis progressive.

*Rabbit 46.*—Age 5 wks. Weight 380 gm. 3rd generation. 0.25 cc. of virus of Rabbit 27 intracranially. Incubation period 10 days. Death in 1 day. Died in convulsions.

*Rabbit 15.*—Age 6 wks. Weight 520 gm. 3rd generation. 0.5 cc. of virus of Rabbit 35 intracranially. Incubation period 2 days. Symptoms progressed rapidly; respiratory distress and great weakness. Chloroformed.

*Rabbit 9.*—Age 4 wks. Weight 440 gm. 3rd generation. 0.5 cc. of virus of Rabbit 35 intracranially. Incubation period 14 days. Death in 7 days. Progressive paralysis with respiratory distress.

*Rabbit 16.*—Age 4 wks. Weight 430 gm. 4th generation. 0.5 cc. of virus of Rabbit 15 intracranially. Incubation period 13 days. Death in 8 days. Symptoms progressive in type with dyspnea.

*Rabbit 33.*—Age 6 wks. Weight 620 gm. 4th generation. 0.5 cc. of emulsion of virus of Rabbit 9, half the amount dropped in each nostril. Incubation period 2 days. Death in 1 day. Symptoms of the fulminating type with paralysis of hind legs towards the last.

*Rabbit 41.*—Age 5 wks. Weight 560 gm. 5th generation. 0.5 cc. of emulsion of virus of Rabbit 33 intracranially. Incubation period 12 days. Death in 4 days. Symptoms of progressive type.

*Rabbit 36.*—Age 4 wks. Weight 380 gms. 5th generation. 0.25 cc. of heavy emulsion of virus of Rabbit 33 in each nostril. Incubation period 2 days. Death in 2 days. Symptoms of fulminating type with paralysis.

*Rabbit 43.*—Age 5 wks. Weight 450 gm. 6th generation. 0.5 cc. of emulsion of virus of Rabbit 36 injected into sciatic nerve sheath. Incubation period 8



days. Symptoms progressive in type. Complete paralysis on 3rd day. Chloroformed.

*Rabbit 49.*—Age 4 wks. Weight 310 gm. 7th generation. 0.5 cc. of emulsion of virus of Rabbit 43 into sciatic nerve sheath. Incubation period 6 days. Death in 2 days. Symptoms fulminating in type.

*Rabbit 64.*—Age 4 wks. Weight 400 gm. 8th generation. 0.25 cc. of emulsion of virus of Rabbit 49 into each nostril. Incubation period 2 days. Death in 2 days. Symptoms of fulminating type.

*Rabbit 61.*—Age 4 wks. Weight 340 gm. 8th generation. 1.0 cc. of emulsion of virus of Rabbit 49 into sciatic nerve sheath. Incubation period 20 days. Died in night with no observed symptoms.

#### DISCUSSION AND SUMMARY.

The poliomyelitic virus obtained from an experimental monkey has been passed through eight generations in rabbits. It shows no signs of dying out. On the other hand, it gives no evidence of becoming more pathogenic to the species through successive passage. The period of incubation remains variable and the percentage of takes has not increased. Whether eventually a virus can be obtained which is of heightened virulence to rabbits is problematic.

All inoculations are by no means successful. The animals show great individual differences in susceptibility to the virus, as is evidenced by the fact that out of fifty-four rabbits inoculated, only twenty-two, or about 40 per cent, succumbed. This fact may explain the negative results of other investigators. At several points in the series of experiments it was thought that the strain had died out. As many as six rabbits have been inoculated one after the other before the virus would catch again.

The age of the rabbits is important in considering the susceptibility. From the limited data at our command, adult rabbits are resistant, and there appears to be an abrupt increase in resistance between the age of 6 and 8 weeks; that is, rabbits under 6 weeks are more susceptible to the virus. There seems to be a parallel between the age incidence of this disease in rabbits and spontaneous poliomyelitis in man. The age incidence of poliomyelitis in man is indicated by the term "infantile paralysis."

Several methods of inoculation have proved successful; thus the rabbits have succumbed as a result of introducing the virus directly

into the brain, by injecting it into a peripheral nerve, or directly into the circulation, or by placing it upon the uninjured nasal mucosa.

The symptoms produced show more or less departure from the symptoms of poliomyelitis as seen in the spontaneous disease in man and in the experimental disease in the monkey. There are two distinct pictures recognizable. In one there is paralysis of one or more of the extremities which progresses until death, resembling somewhat the symptoms of the experimental disease in the monkey. This we have designated the progressive type. The other group is included in what we have called the fulminating type. The symptoms are explosive in character, with extreme weakness amounting to prostration, terminating in death in a few hours, attendant upon respiratory failure. The mode of inoculation seems to have little effect upon the type of symptoms produced.

The period of incubation is variable and apparently does not depend upon the method of inoculation. The period varied from 2 to 41 days, with an average of 12 days. The two extremes both occurred after intracranial injection. After intranasal insufflation the incubation period was short, being in each case 2 days, followed by symptoms of the fulminating type. The placing of the virus into the nose seems to be an effective method, but is as uncertain as other routes, as only three out of nine rabbits tested in this manner succumbed. The disease produced by this route was particularly virulent.

The virus shows no tendency to become fixed. The period of incubation is as variable in the eighth generation as in the first, and the virus has shown no tendency towards increasing virulence through successive passage, in these respects differing from the virus of rabies.

We have found the virus to be filterable. An emulsion of the central nervous matter of a rabbit of the first generation passed through a Berkefeld filter, and injected intracerebrally into another rabbit, resulted in death, preceded by symptoms of the fulminating type. Virus (unfiltered) from this rabbit was transferred successfully to two other rabbits.

The lesions, while definite and consistent throughout the series, lack the distinctive features of the pathologic picture of poliomyelitis in man and the monkey. Capillary congestion, punctate hemor-

rhages, degeneration of the motor cells, satellitosis, and more or less cellular infiltration of the gray matter of the cord and medulla are found, but perivascular infiltration is absent and the infiltrating cells are not lymphocytic in character.

One of the most striking features of this investigation is the way in which rabbits and monkeys react to the same virus. The disease in the rabbit presents certain clinical resemblances to the experimental disease in the monkey and also to the spontaneous disease in children. On the other hand, the symptoms show marked variation from those seen in the monkey and in man. The picture has not the same constancy in rabbits and could not in most cases be recognized clinically as poliomyelitis. There are still more marked differences in the pathology. While it is true that the brunt of the attack in the rabbit falls upon the gray matter of the cord and medulla, the appearance of the lesions under the microscope shows such differences from the lesions of experimental poliomyelitis in monkeys, as well as the natural disease in man, as to suggest two distinct infections. It is more reasonable, however, to assume that we are dealing with a modified form of poliomyelitis; that the rabbit reacts differently to the virus than the monkey or man; and that the disease produced in rabbits by us and others is in fact poliomyelitis. So far as we know, no other virus produces such differences in two animal species. Smallpox is so profoundly altered in the cow that it took almost 100 years to prove Jenner's assumption that cowpox is a modified form of smallpox. However, the pock of vaccinia is a correct counterpart both clinically and pathologically of the pock of variola. If the virus of poliomyelitis may be so altered in the rabbit as scarcely to be recognizable, may it not be still more profoundly changed in other animals? The conjecture then arises that poliomyelitis, instead of being limited naturally to man and experimentally to monkeys, may in fact occur in other animals in unnoticed or unrecognized form. If this should prove true, it may be a source of human infection and may help to solve the problem of prevention.

EXPLANATION OF PLATES.<sup>11</sup>

## PLATE 77.

FIG. 1. Rabbit 19. Paralysis of extremities.

FIG. 2. Rabbit 19. Medulla. Chromatolysis of the nerve cells.  $\times 515$ .

## PLATE 78.

FIG. 3. Rabbit 9. Lumbar cord, showing capillary congestion and hemorrhage.  $\times 180$ .

FIG. 4. Rabbit 9. Lumbar cord. Numerous punctate hemorrhages, and marked cell degeneration.  $\times 385$ .

## PLATE 79.

FIG. 5. Rabbit 35. Lumbar cord. Cellular infiltration.  $\times 180$ .

FIG. 6. Rabbit 9. Medulla. Satellitosis around degenerated nerve cells.  $\times 515$ .

## PLATE 80.

FIG. 7. Rabbit 35. Satellitosis.  $\times 515$ .

FIG. 8. Rabbit 19. Cervical cord. Degeneration of anterior horn cells with satellitosis.  $\times 385$ .

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<sup>11</sup> We are indebted to Dr. J. P. Bill for the microphotographic work.

FIG. 1

FIG. 2.

(Rosenau and Havens: Poliomyelitis in the Rabbit.)



FIG. 3.

FIG. 4.

(Rosenau and Havens: Poliomyelitis in the Rabbit.)





FIG 5

FIG 6

(Rosenau and Havens: Poliomyelitis in the Rabbit.)



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FIG. 7.

FIG. 8.

(Rosenau and Havens: Poliomyelitis in the Rabbit.)



FIG. 7.

FIG. 8.

(Romanau and Havens: Poliomyelitis in the Rabbit.)



## FEEDING EXPERIMENTS WITH BACTERIUM PULLORUM. THE TOXICITY OF INFECTED EGGS.

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In a previous investigation on the bacteriology of the hen's egg<sup>1</sup> sufficient evidence was acquired to show that the contents of normal fresh eggs are, as a rule, sterile, and that even eggs which have been incubated artificially for 3 weeks remain relatively free from bacterial invasion, provided that they were fresh and clean when placed in the incubator. It was shown, however, that eggs which come from fowls that are permanent bacillus (*Bacterium pullorum*) carriers are often exceptions to the rule.

The carrier problem in bacillary white diarrhea of young chicks has assumed considerable importance in recent years.<sup>2</sup> It has been demonstrated beyond doubt that the mother hen is the permanent source of infection, as the result of acquiring the disease early in her existence, or after full maturity. The permanent seat of infection is the ovary, which in many instances becomes so greatly involved that the ova are discolored and misshapen, and the ovary presents a decidedly pathological appearance. The ova harbor the disease organism. Furthermore, ova which develop into apparently normal yolks frequently carry the organism, *Bacterium pullorum*, to the time of full formation of the egg. Infected eggs produce infected chicks, and although chicks succumb frequently before their embryonic development is completed, a large percentage of them emerge from

<sup>1</sup> Rettger, L. F., *Centralbl. f. Bakteriol., 2te Abt.*, 1913-14, xxxix, 611; *Bull. Storrs Agricultural Experiment Station*, 1913, No. 75.

<sup>2</sup> Rettger, L. F., and Stoneburn, F. H., *Bull. Storrs Agricultural Experiment Station*, 1909, No. 60; 1911, No. 68. Rettger, L. F., Kirkpatrick, W. F., and Stoneburn, F. H., *Bull. Storrs Agricultural Experiment Station*, 1912, No. 74. Rettger, L. F., Kirkpatrick, W. F., and Jones, R. E., *Bull. Storrs Agricultural Experiment Station*, 1914, No. 77.

the shell apparently sound and well. However, they soon acquire the disease and many of them fall a prey to the organism which they carried for a long time in their yolk. Those which survive often become permanent bacillus carriers, and thus the cycle of infection is completed. Furthermore, these chicks are a constant source of infection to other chicks and mature stock as well.

The occurrence of *Bacterium pullorum* in eggs has been a subject of serious consideration, but only from the standpoint of breeding and the perpetuation of sound stock. The system of testing breeding hens by the agglutination test, which is now being conducted in several of the states, marks but a culmination of the efforts that are being made to combat the disease through the detection and the elimination of the permanent carriers of bacillary white diarrhea.

There has been much speculation from time to time as to whether the presence of *Bacterium pullorum* in an egg renders it unsafe as an article of diet for man or any of the lower animals other than chicks, and especially for young children and infants. Little serious thought was given to this question, however, and it was dismissed with the assumption that such an element of danger, if it existed at all, was but very slight. While it is not the purpose of this paper to spread undue alarm, there is sufficient evidence on hand to show that *Bacterium pullorum*, when fed to some of the lower animals, may produce most serious consequences, and even death. In other words, it has been demonstrated that this organism may manifest itself as one of the group of so called "food-poisoning bacteria."

The marked pathogenicity of *Bacterium pullorum* for young chicks has been so frequently demonstrated that little need be said concerning it here. Chicks under 1 week of age are extremely susceptible to the influence of this organism, whether infection takes place through the mouth or the skin (subcutaneous injection), and the mortality is usually very high. It had long been supposed, however, that the ability of this organism to produce disease did not extend beyond young chicks.

Jones<sup>3</sup> was the first to demonstrate that *Bacterium pullorum* may be pathogenic for fully mature fowls. On a large poultry farm in the state of New York

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<sup>3</sup> Jones, F. S., *Jour. Med. Research*, 1912-13, xxvii, 471.



a peculiar epidemic manifested itself in a flock of about 700 adult hens, the resultant mortality being 50. This epidemic was most clearly traced to infected eggs, as the following account will show. There had been no history of bacillary white diarrhea on the farm. A neighbor had been meeting with serious losses, however, and a number of eggs from his fowls were incubated on the farm which had hitherto been free from the disease. Nearly all the chicks that were hatched from these eggs died within 10 days from bacillary white diarrhea. The eggs which failed to hatch were inadvertently fed on Mar. 12 to the adult hens. On Mar. 28 a few of the fowls which had eaten the eggs died, and from that time on for a period of a month 50 of the hens died, with essentially the same symptoms. *Bacterium pullorum* was recovered from various internal organs of hens that were examined, as well as from chicks hatched from the neighbor's eggs, and from the ovaries of the neighbor's adult fowls.

We have obtained similar results, though on a much smaller scale. In a series of experiments conducted in 1913 on mature fowls, and in which bouillon cultures of *Bacterium pullorum* were fed to the fowls along with the regular dry mash, three deaths occurred in a pen of twelve hens. Two of the hens were sent to the laboratory for bacteriological examinations. From the liver, lungs, spleen, and heart of both these victims *Bacterium pullorum* was easily recovered, and the growths obtained on agar by direct inoculation were such as to indicate that the bacilli were present in the blood in very large numbers. Postmortem examinations did not reveal any marked or gross lesions. The deaths occurred within a period of 1 to 2 weeks after the first ingestion of the bouillon cultures. Other hens which appeared to be affected completely recovered in a relatively short time.

The first information as to the real toxicity of *Bacterium pullorum* for experimental animals was conveyed by Smith and Ten Broeck.<sup>4</sup> In comparative studies of the properties of this organism and of the bacillus of fowl typhoid these authors demonstrated that the bacterium-free filtrates of 5 to 15 day old bouillon cultures of *Bacterium pullorum* were decidedly toxic to full grown rabbits when given by intravenous injection. Death followed within 2 hours, or there was marked dyspnea followed by death over night or by loss of weight and subsequent recovery.

Gage<sup>5</sup> has shown that rabbits are very susceptible to even very small doses of *Bacterium pullorum* by subcutaneous injection, and that for this reason it is difficult to immunize rabbits to the organism.

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<sup>4</sup> Smith, T., and Ten Broeck, C., *Jour. Med. Research*, 1915, **xxi**, 547.

<sup>5</sup> Gage, personal communication.

The present investigation divides itself into two distinct phases: first, a study of the toxicity or disease-producing properties of *Bacterium pullorum* when administered orally, either with food or with the aid of a pipette; and, second, an investigation of the heat tolerance of this organism in infected eggs which are being prepared for table use by the usual processes of boiling, coddling, frying, etc.

*The Toxicity of Bacterium pullorum when Introduced through the Mouth and Digestive Tract.*

The experiments were conducted on 22 rabbits, 11 kittens, 11 guinea pigs, and 4 white rats. The method of administering the test organism varied. In some instances water suspensions of slant agar cultures were mixed with the regular feed, while at other times these suspensions were given with a pipette. In a number of the experiments the yolks of eggs were employed which had been artificially infected with pure cultures of the organism by injecting relatively small numbers directly into the yolk, through the shell and white, and incubated for at least 2 or 3 days. The different animals were weighed at frequent intervals, usually each day, and their general condition was observed. Those that died were subjected to post-mortem examination, with a special effort to determine the presence or absence of *Bacterium pullorum* in the blood of the internal organs.

In Table I are given the results of the infection experiments with rabbits, in so far as the weights and deaths are concerned. It will be seen that the initial weights of the animals varied from 375 to 2,455 gm., the majority of the rabbits being half grown or smaller. More complete data are given in the individual records following the table.

Rabbits 1 and 2 were fed large amounts of suspensions of five different strains of *Bacterium pullorum* (1 to 5 cc.) daily. On the 5th day they refused to eat, and died on the 7th and 6th days. Pure cultures of *Bacterium pullorum* were obtained from the internal organs. Rabbits 3, 4, 5, and 6 received one large dose of the organism with the food. From the blood of each of these animals the bacterium in question was recovered in large numbers, after death. Rabbits 7 and 8 were given the surface growth of one tube of slant agar. Blood



tests were again positive. Rabbit 9 was fed 0.1 of a slant agar growth. Death occurred on the 7th day. *Bacterium pullorum* was demonstrated in the blood.

Rabbit 10 received 0.3 of a 24 hour slant agar culture. It continued to gain weight for 40 days. It was then given 0.5 cc. of a mixture of three strains, but continued to gain for 2 weeks. A third dose of 0.7 cc. likewise had no visible effect.

Rabbit 12 served as a control animal, and was therefore not subjected to an infection experiment. Aside from this rabbit there were at all times at least two or three rabbits which belonged to the same lots as the test animals, and which were kept under practically the same conditions, except for the administration of suspensions of *Bacterium pullorum*. None of these stock rabbits died or showed any indications of illness.

Aside from a loss of 65 gm., Rabbit 13 was apparently unaffected after receiving through a pipette 2 cc. of a suspension obtained by washing three slant agar cultures.

Rabbits 14 and 15 received 0.5 and 1.0 cc. respectively of the yolk of incubated eggs which had been artificially infected with *Bacterium pullorum*. Rabbits 16, 17, 18, and 19 were given with a pipette 1.5, 2.0, 3.0, and 5.0 cc. of the yolk of artificially infected eggs. In addition to losses in weight, and death in each instance, there were indications of a diarrheal condition. With the exception of No. 18, blood tests with each of these animals for *Bacterium pullorum* gave positive results.

Rabbits 20 and 21 were kept as controls in separate cages. They remained apparently normal.

Rabbit 22 was a good sized adult male which had been suffering from a large abdominal abscess. Although the abscess was lanced it continued visibly to affect the health of the animal. 10 cc. of the yolk of an infected egg were given by mouth. On the 5th day there were marked diarrheal symptoms, and on the 6th day the rabbit died. *Bacterium pullorum* was recovered from the heart, liver, and lungs in large numbers.

Postmortem examination of the rabbits which died apparently from the effects of infection with *Bacterium pullorum* revealed no gross

lesions or other marked pathological condition. The small intestine was usually empty and decidedly pale. At times a light yellow viscid fluid was present in the lumen. In the lower intestine the contents were often less firm than in the normal rabbit, and frequently there was evidence of a diarrheal condition.

The liver was more or less congested. The surface was often marked by minute areas which appeared to be of a necrotic character. Aside from some congestion the spleen and kidneys were to all appearances normal. No pathological condition of the heart and lungs was visible to the unaided eye. Since no investigation was made of the histological structures of the different organs no details can be given as to their minute pathology.

The internal organs of the cats that died showed no marked pathological appearances, and to a large extent resembled those of the rabbits that succumbed to infection. Congestion was apparent, especially in the liver. Culture tests upon Cats 1, 2, 6, 7, and 8 gave positive results, *Bacterium pullorum* being recovered with ease from the blood of the liver, kidney, and lungs, except in No. 7 in which only the lungs contained the organism in question in sufficient numbers to obtain positive cultures. Agglutination tests with positive fowl sera proved the organism to be *Bacterium pullorum*.

The early death of the control animal was either due to some other cause or agent, aside from *Bacterium pullorum*, or was the result of rapid infection of this kitten from the vomited matter and diarrheal discharges of other kittens that were the first to be seriously affected. These experiments on kittens are to be repeated, with enough controls kept in separate cages to make the results as conclusive as possible.

*White Rats.*—Four white rats weighing from 100 to 250 gm. were used in these experiments. They were given one large dose of *Bacterium pullorum* in a water suspension. None of the animals showed any signs of discomfort or illness after the treatment, and all continued to increase in weight for the entire observation period of 24 days. The rats, although small as compared with adults, were apparently unaffected by one treatment with the organism (3 to 5 cc. of the suspension).

TABLE II.  
Cats. Weight in Gm., and Mortality Records.

Cat No.	Initial weight.	1	2	3	4	Days after first administration of <i>Bacterium pullorum</i> by mouth.							14	20	23	40
1	1,088	—	1,180	1,160	—	1,192	—	1,115	1,074	985	915	790	Dead.			
Given 2.0 cc. of suspension of <i>Bacterium pullorum</i> (with pipette) at beginning of experiment.																
2	1,098	—	1,158	1,136	—	1,200	—	1,130	997	955	890	750	“			
Given 1.0 cc. of suspension.																
3	928	—	940	920	—	846	—	805	776	755	750	810	795	Dead.		635
Given 0.5 cc. of suspension.																
4	618	—	632	—	—	535	492	Dead.								
Received 0.5 cc. of suspension of <i>Bacterium pullorum</i> in water.																
5	600	—	600	—	—	590	636	535	525	530	Dead.					
Given 1.0 cc. of suspension.																
6	590	—	590	—	—	485	492	Dead.								
Given 1.5 cc. of suspension.																
7	660	—	680	—	—	615	620	575	595	Dead.						
Given 2.0 cc. of suspension.																
8	620	—	820	—	—	800	710	Dead.								
Control. Not artificially infected, but kept in same enclosure as Cats 4 to 7.																
9	990	935	910	895	885	880	—	795	770	770	685	700	710	Alive.		
Received 0.5 cc. of suspension.																
10	990	1,000	965	980	980	940	—	990	925	905	865	880	890	“		
Given 1.0 cc. of suspension.																
11	730	740	746	725	745	705	—	710	685	695	690	650	Alive.			
Given 0.5 cc. of suspension.																

Kittens 1 to 3 were from one litter, Nos. 4 to 8 from another, and Nos. 10 and 11 from a third. While No. 8 was not artificially infected she was allowed to be in contact with Nos. 1 to 7 which were fed suspensions of *Bacterium pullorum* with a pipette. As vomiting and purging occurred very soon in these cats, the possibility of the control's becoming infected early cannot be excluded.

Kittens 4 to 7 were fed the bacterial suspensions 3 days later than Nos. 1 to 3. Signs of disturbances became apparent in both groups at about the same time. Vomiting, diarrhea, and rapid loss in weight were the most marked symptoms in the animals that died. Vomiting and diarrhea were most acute. The temperature during the time of the most severe attacks was decidedly subnormal, and for several days before death the kittens were extremely weak and listless. Cats 9, 10, and 11 showed no signs of illness except loss in weight.

TABLE III.

*Guinea Pigs. Weight in Gm., and Mortality Records.*

Guinea pig No.	Initial weight.	1	2	3	4	5	6	8	10	12	14	16	18	20
Days after first administration of <i>Bacterium pullorum</i> by mouth, and of additional doses.														
1	540	—	—	560	—	540	525	528	529	525	515	510	510	538
Bacterial suspension of <i>Bacterium pullorum</i> mixed with food at beginning of experiment.														
2	510	—	—	512	—	502	495	482	512	498	505	518	514	555
Same treatment as No. 1.														
3	717	718	710	710	700	723	702	703	—	694	710	698	705	740
Same treatment as No. 1.														
4	365	370	385	—	360	340	325	Dead.						
Given varying amounts of infected yolk daily for 6 days (1 to 5 cc. of yolk.)														
5	75	78	80	85	84	—	83	Dead.						
Given 0.5 cc. of heavy bacterial suspension at beginning of experiment.														
6	365	370	385	360	340	325	330	275	Dead.					
3cc. 3cc. 5cc. 8cc. — 3cc. 8cc. of infected yolk, given on respective dates.														
7	425	425	420	405	405	410	400	415	430	—	—	—	—	Dead.
7cc. 5cc. 6cc. 5cc. 4cc. of infected yolk.														
8	315	—	310	—	330	315	—	330	—	335	—	—	—	355
10cc. 10cc. of infected yolk.														
9	315	—	310	—	265	285	—	295	—	300	—	—	—	325
10cc. 10cc. of infected yolk.														
10	345	320	Dead.	One feeding, half of an infected yolk.										
11	405	380	355	320	Dead.	One feeding, half of an infected yolk.								

In Guinea Pig 4 a diarrheal condition was apparent on the last 2 days. The liver and kidneys were congested. Other organs were normal. Cultural tests were negative. Guinea Pig 7 showed evidence of marked diarrhea just before death. There was some congestion of the liver and spleen, and hemorrhage in the pericardial sac. Culture tests from the different organs were negative. In Nos. 6, 7, and 10 slight diarrhea was apparent, but no other symptoms, except loss of weight and lessened appetite, were observed.

Cultural tests were again negative. *Bacterium pullorum* was isolated from the liver and lungs of Guinea Pig 11, but not from the blood of any of the other five animals that died.

## DISCUSSION.

The foregoing data on the oral administration of *Bacterium pullorum* can leave no doubt as to the so called toxicity of this organism for young rabbits when given by mouth. The results of the feeding experiments with kittens of different ages are less conclusive, and require further substantiation, in view of the fact that there was no satisfactory control. They are strongly indicative, however, of a most harmful influence exerted by relatively large numbers of the organism when administered by mouth. The symptoms of the disturbance produced in the kittens were those of a food-poisoning organism, especially the vomiting and diarrhea, and the extreme emaciation. The possibility that these symptoms are due to a complication of disturbing factors, cannot be entirely ignored, however.

Adult guinea pigs are but slightly susceptible to *Bacterium pullorum*, when ingested, though six of the eleven animals employed died, some early and others late in the periods of observation. With but one or two exceptions, they were given large amounts of the organism. Further experiments with guinea pigs are now in progress. Rats, according to the above data, are immune to disturbing influences of *Bacterium pullorum* when it is given by the mouth, even in large numbers.

Although there are no cases on record of food-poisoning, enteritis, or other ailments in man which have been ascribed to *Bacterium pullorum*, the possibility of danger from infection with this organism can no longer be ignored, especially in so far as invalids and young children are concerned. Eggs are among the most common articles of diet, and for the sick and convalescent as well as for children of all ages they often rank with milk as one of the most important foods. Not only are they consumed soft boiled, but they are frequently prescribed in the raw state.

Fresh eggs, when they are infected with *Bacterium pullorum*, contain this organism in such small numbers as to constitute no real danger of disturbance even for the most infirm, or for the smallest infants. It has been conclusively demonstrated, however, that the organism multiplies very rapidly in the yolks of infected eggs, when



the eggs are held at or near so called incubator temperature.<sup>6</sup> Hence, eggs which are left in the nest under broody hens for but comparatively few hours, or which are not kept in cool places during storage and transportation, especially during the warm summer months, harbor the organism in large numbers. In fact, they are so abundant that the inoculation of the surface of slant agar with but a small portion of the yolk held in a platinum loop results in the production of an almost solid surface growth on the agar.

It has also been shown that in various sections of this country a large percentage of the flocks and of the individual hens are permanent carriers of white diarrhea (*Bacterium pullorum*) infection. In the survey of the conditions in Connecticut it was found that out of 107 flocks which were tested for ovarian infection with *Bacterium pullorum* 79, or 74 per cent, possessed bacillus carriers, and of a total of 13,831 fowls that were subjected to the agglutination test 1,417, or 10.24 per cent, gave positive indications of ovarian infection.<sup>7</sup> In an investigation recently conducted in Massachusetts similar results were obtained.<sup>8</sup> In some flocks over 50 per cent of the individual fowls that were tested were reactors. The same condition undoubtedly prevails throughout a large part of the Country, though no definite figures have been obtained outside of Connecticut and Massachusetts.

The common methods of boiling or frying infected eggs for the table do not necessarily render the eggs sterile, in so far as *Bacterium pullorum* is concerned, as the following data will show.

*The Survival of Bacterium pullorum in the Yolks of Eggs after Various Methods of Treatment with Heat. The Influence of Cooking.*

These experiments were carried on with eggs which were artificially infected with *Bacterium pullorum* in the following manner. Fresh

<sup>6</sup> Rettger and Stoneburn, *Bull. Storrs Agricultural Experiment Station*, 1911, No. 68. Rettger, Kirkpatrick, and Jones, *Bull. Storrs Agricultural Experiment Station*, 1914, No. 77. Rettger, *Centralbl. f. Bakteriologie*, 2te Abt., 1913-14, xxxix, 611.

<sup>7</sup> Rettger, Kirkpatrick, and Jones, *Bull. Storrs Agricultural Experiment Station*, in press.

<sup>8</sup> Gage, G. E., and Paige, B. H., *Bull. Massachusetts Agricultural Experiment Station*, 1915, No. 163.

eggs were immersed for a few minutes in alcohol, and one of the ends was flamed. A small hole was made through the shell without injuring the shell membrane. The eggs were then inoculated by injecting about 0.25 cc. of a water suspension of the organism with a sterile hypodermic syringe directly into the yolk. The hole was sealed with collodion and the eggs were incubated for varying lengths of time, usually 3 to 5 days.

Infected eggs were placed in boiling water, and held there for different lengths of time. They were then chilled in cold water and opened aseptically. Small amounts of yolk were streaked over the surface of slant agar and the tubes incubated at 37°C. The results are given in Table IV.

TABLE IV.

Time of boiling.	Coagulation of white.	Thickening of yolk.	Growth of <i>Bacterium pullorum</i> on slant agar.
min.			
1.....	0	0	+
2.....	0	0	+
2½.....	Slight.	0	0 + + + + +
3.....	+	Slight.	0 0 0 + + + +
3½.....	+	+	0 0 0 0 0 + + + +
4.....	+	+	0 0 0 + +

In the last column 0 indicates no growth, and + the characteristic growth of *Bacterium pullorum*. Each 0 and + sign represents an individual egg. The variation in the results of the different eggs is undoubtedly due to differences in the size of the eggs and the thickness of the shells, and perhaps in part to the differences in the numbers of bacteria in the eggs.

Poaching the eggs for  $\frac{1}{2}$  to 4 minutes rendered them sterile. Artificially infected eggs that were scrambled were likewise found to contain no viable organisms. On the other hand, fried and coddled eggs gave varied results, as will be seen in the following brief summaries.

In all the foregoing experiments control tests were made with eggs that had been inoculated and incubated in the same way as the others. No difficulty was experienced in obtaining an abundance of the bacilli in the yolks after the various periods of incubation of the eggs.

It should be stated that all these experiments were conducted at an altitude of 700 feet above sea level, where the boiling point of water is 99.2°C.

TABLE V.  
*The Effect of Frying.*

Physical character of heated eggs.	Growth on slant agar.
Frying on one side only.	
Soft.	+ + + +
Medium.	+ +
Hard.	+
Frying on both sides.	
Soft.	0 0
Medium.	0 0
Hard.	0 0

TABLE VI.  
*The Effect of Coddling.*

Time of heating. min.	Growth on slant agar.
3	0 +
3½	0 0
4	0 + + +
4½	+
5	0 0
7	0
10	0

The eggs were coddled by pouring boiling water over them in a granite receptacle and allowing them to stand away from a flame or stove for the different lengths of time. About 1 quart of water was employed for each egg.

The experiments on the viability of *Bacterium pullorum* in egg yolk after varying periods of heating show that the organism under the stated conditions possesses a high degree of resistance. Even boil-

ing for 4 minutes did not in every instance destroy it. This resistance is due undoubtedly to the peculiar protection which is afforded, first by the shell, then by the egg white, and finally by the yolk itself. The high per cent of fat in the yolk is undoubtedly an important factor.

It has been demonstrated by Smith<sup>9</sup> and others that tubercle bacilli are less readily killed by the ordinary process of pasteurization when they are held in the film or pellicle layer of milk than in the whole or mixed milk.

Other organisms of the colon-paratyphoid-typhoid group would undoubtedly show the same resistance under similar conditions of environment. Chantemesse and Rodriguez<sup>10</sup> report an epidemic of food-poisoning which was due to cream cakes. The meringue of the cakes was found to contain a peculiarly toxic microorganism of the *Bacillus paratyphi* type, and although the meringue had been heated to browning numerous bacilli of this type were found in the interior portion. Quite recently a small epidemic of typhoid fever was pronounced to have been caused by the consumption of baked spaghetti.<sup>11</sup> The typhoid bacillus was readily identified in the inner portion of spaghetti that was artificially inoculated and baked, in spite of the fact that the dish was subjected to sufficient heat to brown the surface and to char the protruding ends of the spaghetti sticks.

#### SUMMARY AND CONCLUSIONS.

The problem of eradicating ovarian infection in the domestic fowl assumes still greater importance than heretofore, in the light of data recently acquired. Not only is it of great significance to eliminate the permanent carriers of *Bacterium pullorum* from all flocks of fowls from the standpoint of successful poultry breeding, but also because they constitute a possible source of danger to man.

Eggs which harbor *Bacterium pullorum* in the yolk in large numbers may produce abnormal conditions, when fed, not only in young chicks, but in adult fowls, young rabbits, guinea pigs, and kittens. The toxicity for young rabbits is most pronounced, the infection usually resulting in the death of the animals. In kittens the most prominent symptoms are those of severe food-poisoning with members of the paratyphoid group of bacteria. The possibility of infected eggs causing serious disturbances in young children and in the sick

<sup>9</sup> Smith, T., *Jour. Exper. Med.*, 1899, iv, 217.

<sup>10</sup> Chantemesse and Rodriguez, *Bull. Acad. de méd.*, 1914, lxxi, 245; Abstract in *Experiment Station Record*, 1914, xxxi, 555.

<sup>11</sup> Johnston, H., *Health News*, 1915, xxxi, 173.

and convalescent of all ages must therefore receive serious consideration.

Ovarian infection of fowls is very common throughout this country. Hence, a large proportion of the marketed eggs are infected with *Bacterium pullorum*. When such eggs are allowed to remain in nests under broody hens, or in warm storage places, for comparatively few hours, they contain large numbers of the organism.

Soft boiling, coddling, and frying on one side only do not necessarily render the yolks free from viable bacteria; therefore, eggs which have gone through these processes may, like raw eggs, be the cause of serious disturbances in persons who are particularly susceptible to such influences, and especially to infants.

That no well authenticated instances of egg-poisoning of this kind are on record does not warrant the assumption that there have been no cases. The etiology of infantile stomach and intestinal disturbances is as yet too little understood; in fact, it may be said that many of these disorders have no known cause, and almost as much may be said regarding gastro-intestinal diseases in later life. Furthermore, since the ailments caused by infected eggs would not make themselves felt presumably until several days after their ingestion, little or no suspicion would fall upon the eggs. It may be said, too, that the wide distribution of ovarian infection in the domestic fowl has come about only in the last few years, hence its possible danger to man is one of recent development.



# SERUM CHANGES AND THE CAUSE OF DEATH IN EXPERIMENTAL PANCREATITIS.

## STUDIES ON FERMENT ACTION. XXX.

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Incidental to a study of the ferment balance of the serum during various pathological conditions we have had occasion to observe the serum changes in a series of eighteen dogs in which an acute experimental pancreatitis had been produced.

Various theories have been advanced to account for the marked intoxication resulting from an acute pancreatitis. Opie (1) in this country, and Eppinger (2), Pólya (3), von Bergmann and Guleke (4), and numerous other workers in Europe have fully discussed the problem. The changes in the lipase titer have been reported by Whipple and Goodpasture (5).

Most workers are convinced that an activation (probably intracellular) of the tryptic proenzyme occurs with a resulting intoxication of the animal because of the sudden formation and absorption of toxic split products. Coincident with this change there may occur a saponification of fats due to the simultaneous activation of the pancreatic lipase with a further injury to the pancreatic tissue by the soaps so formed. The means of activation of these ferments, resulting from a primary injury, may, of course, be diverse: bacterial infection, either through the ducts or lymphatics; mechanical blocking of the ducts and activation from bile; or activation from enterokinase and from tissue juice resulting from direct trauma, possibly from injury following vascular changes. While, therefore, the primary factor may be diverse, the resulting pathological lesions and the cause of death are uniform.

In the following illustrative experiments three different substances were injected as activators: bile salts, as first recommended by Flexner (6), active trypsin solutions, and sodium oleate solutions.

These experiments and the charts of the serum changes follow.

*Dog 1.*—Weight 4.5 kilos. 0.2 gm. of bile salts was injected into the pancreatic duct with subsequent ligation, at 9.30 a.m. on May 10, 1915. Serum samples were collected before the operation, at 10 and 11 a.m., and at 1 and 3 p.m. on each of the following 2 days and after about 3 weeks (June 3, 1915). The serum changes are shown in detail in Text-fig. 1.

The animal was killed on June 3, 1915. The pathological changes noted at autopsy were as follows: The pancreatic duct was occluded. The body of the pancreas was atrophic, fibrous, with large areas of fatty change and small necrotic foci containing caseous material. The tail of the pancreas showed less change. The viscera presented no pathological alteration other than fatty changes.

*Dog 2.*—Weight 5 kilos. 1 gm. of sodium oleate was injected into the pancreatic duct with subsequent ligation, at 11.30 a.m. (May 14, 1915). Serum samples were collected before the operation at 11.50 a.m., and at 1.30 and 3.30 p.m. on the 3 succeeding days, and after several weeks (June 4, 1915). The dog was killed after being used in another experiment. The serum changes are shown in detail in Text-fig. 2. The findings at autopsy were as follows: The pancreatic tissue was represented by a small remnant about one-fourth the size of the original tissue, rather firm, and pale yellow in color. The duct was occluded and there were numerous firm adhesions to the adjoining viscera. Scattered throughout the remnants of the gland were small more or less caseous areas of necrosis.

*Dog 3.*—Weight 5.1 kilos. 0.2 gm. of trypsin (purified) was injected into the pancreatic duct, with subsequent ligation, at 9.10 a.m. (June 7, 1915). Serum samples were collected before the operation at 9.45 a.m., noon, and 3 p.m., and at 8.30 a.m. and 2 p.m. the following day. The animal died at 2.15 p.m. (June 8, 1915). The serum changes are shown in detail in Text-fig. 3.

The autopsy findings were as follows: On opening the abdominal cavity a considerable amount of sterile, hemorrhagic, opaque fluid was found. This exudate contained 0.28 mg. of non-coagulable nitrogen per cc., and had a slight antitryptic action. Incubated under toluol the non-coagulable nitrogen increased 0.27 mg., and under chloroform 1.72 mg. in 16 hours, indicating the presence of considerable amounts of proteolytic ferments. The pancreatic tissue was hemorrhagic and contained numerous areas of necrosis throughout; there were numerous areas of fat necrosis scattered over the omentum. Fibrinous adhesions were found about the pancreas and adjacent viscera.

In the first animal the injection of bile salts caused only a moderate pancreatitis, although at the time the abdomen was closed after the injection a thorough infiltration of the pancreatic tissue was noted.





PROTEASE  
1.0 MC  
0.9  
0.8  
0.7  
0.6  
0.5  
0.4  
0.3  
0.2  
0.1

LYMPH  
10 CC  
9  
8  
7  
6  
5  
4  
3  
2  
1

RYTHM  
10  
9  
8  
7  
6  
5  
4  
3  
2  
1

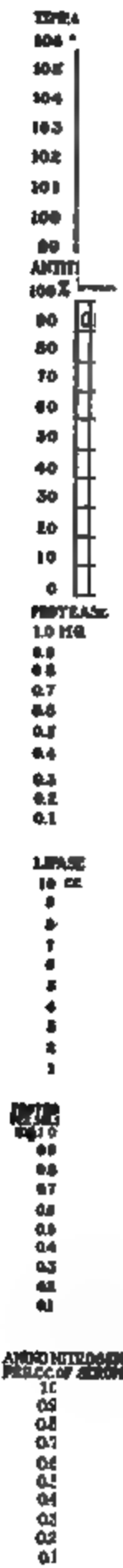
ANTHROPOMETER  
10  
9  
8  
7  
6  
5  
4  
3  
2  
1

TEXT-FIG. 1. Serum changes accompanying acute pancreatitis due to bile salt injections.

1

PENTRASE  
 1.0 ml.  
 0.9  
 0.8  
 0.7  
 0.6  
 0.5  
 0.4  
 0.3  
 0.2  
 0.1  
  
 LIPID  
 10 mg.  
 9  
 8  
 7  
 6  
 5  
 4  
 3  
 2  
 1  
  
 TIME  
 1.0  
 0.9  
 0.8  
 0.7  
 0.6  
 0.5  
 0.4  
 0.3  
 0.2  
 0.1  
  
 AND NITROGEN  
 10 mg.  
 0.9  
 0.8  
 0.7  
 0.6  
 0.5  
 0.4  
 0.3  
 0.2  
 0.1

TEXT-FIG. 2. Serum changes accompanying acute pancreatitis due to soap  
 injections.



TEXT-FIG. 3. Serum changes accompanying acute pancreatitis due to trypsin injections.

No increase in temperature resulted; only a moderate leukocytosis and that only for a short period of time. Immediately after the operation the antiferment increased while the protease decreased; the balance was restored to a normal level the following day. The serum lipase increased slightly. There was noted a marked increase in serum proteoses during the period following the operation; after 3 weeks a normal value was again found. The amino nitrogen in this, as in the other animals, showed only very slight changes.

In the third dog the injection of the trypsin resulted in a rapidly fatal intoxication and it will be observed that the serum changes were different. There was a marked leukocytosis—80,000—as an index of the intoxication, with a progressive decrease in temperature. The antiferment, after a short rise, declined progressively, while the serum protease increased and remained high. The proteoses, after a slight initial decrease, frequently observed in the period following the mobilization of protease, increased markedly. There was only a slight change in the lipase.

The picture closely resembles the condition found in true trypsin shock (7), except in the behavior of the serum lipase. In conjunction with Experiment 2 it would seem that the decrease in the antiferment and the increase in protease have had some relation to the fatal outcome in this case.

In the second dog in which soap was injected, we find the reverse of the preceding changes, as might be expected from the difference in the action of the substances. The tissue destruction was quite marked and the whole pancreas even before closing the abdomen presented a deeply engorged, hemorrhagic, semitranslucent mass almost twice the normal size. It will be observed that the leukocyte count was also high—100,000 after 24 hours. Despite the severity of the local process the serum changes were slight and the animal showed little evidence of a general intoxication. The antiferment increased immediately, but the serum picture was practically unaltered after 24 hours. The lipase remained low. As contrasted with the two previous animals it will be seen that the increase in proteoses was not only delayed but actually less in amount.

## DISCUSSION.

From the series of animals, of which these three are representative, we believe that we are justified in assuming that death is due to the sudden flooding of the blood stream with the higher split products formed at the expense of the pancreatic tissue, of which the proteose increase is an index. Except in the experiments in which active trypsin is used for injection there is no increase in serum protease at any time, as would be expected if the intoxication were a true trypsin shock, nor is there much change in serum lipase (esterase), the condition in this respect resembling closely the results observed following the injection of protein split products (8).

Here, as in other protein intoxications (9) the increase in antiferment seems to be of distinct value in the protection of the animal; in the experiment in which soap was used for the injection this becomes apparent. The increase in antiferment was marked, while the delay in the digestion of the pancreas and the consequent lessening of the shock is indicated by the gradual and relatively small increase in the amount of proteoses present in the serum.

In view of Whipple's results concerning the non-toxicity of the abdominal exudate (5) and our own experiments, it would seem that the only beneficial effects which might possibly have accrued from operative interference in cases of human pancreatitis have resulted from the prolonged anesthesia rather than from the surgical drainage. The inhibitory effect of anesthetics on the development of toxicity for protein intoxication is well known.

## CONCLUSIONS.

1. The serum changes observed during acute experimental pancreatitis indicate that the shock and death are due to an intoxication from protein split products, and not to an intoxication from pure tryptic ferment.

2. When the pancreatitis is produced by the injection of an anti-proteolytic substance (sodium oleate), the degree of intoxication bears no relation to the degree of tissue destruction.

3. The increase in serum antiferment apparently favors the recovery of the animal.

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## SERUM CHANGES FOLLOWING THYROPARATHYROID-ECTOMY.

### STUDIES ON FERMENT ACTION. XXXI.

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As a result of the work of MacCallum and his coworkers (1), our knowledge concerning disturbances of the inorganic constituents of the blood serum particularly, following the removal of the parathyroids has been greatly advanced, and the antagonistic effect of the calcium ions established.

Certain observations have accumulated, largely of a clinical nature, which seem to indicate that in the tetany of infancy dietary factors are of considerable importance both from an etiological and therapeutic point of view. The spasmophilic infant rapidly recovers on a proper diet, and the evidence seems to indicate that phosphorus and certain of the unsaturated oils are of aid in expediting a favorable outcome.

In adults, too, we have to consider the peculiar form of tetany associated with gastric and occasionally intestinal disturbances, under conditions, therefore, which may be considered to offer opportunities for the absorption of the higher split products of proteins, the gastric digestion, of course, only splitting through to the peptones.

Experimentally the observation has been frequently recorded that the feeding of meats is particularly prone to lead to the onset of tetany in parathyroidectomized dogs.

Considerations such as these probably led Kling (2) to study the electric excitability of animals during anaphylaxis, in an effort to correlate the increased irritability of tetany with an anaphylactic state depending on a protein intoxication.

Our knowledge concerning the pathological effects of a faulty absorption of the higher split products of proteins from the gastro-intestinal tract is as yet fragmentary, despite the obvious clinical evidence pointing in this direction. It has been observed, however, that in gastric tetany a gastro-enterostomy usually relieves the condition, the operative interference probably enabling a more rapid emptying of the stomach to the intestine with an augmented splitting of the protein contents to lower stages. Kaufmann's work is of particular interest in this connection (3).

In the few cases of true intestinal tetany that have been reported, there is considerable evidence that an achylia gastrica frequently complicates the picture, which again would offer every opportunity for a rapid absorption of the higher split products from the intestine, which under normal conditions is only afforded an opportunity to absorb the lower split products (Fleiner (4), Quosig (5)). The relation of tetany in infants is of particular interest in this connection; for substances which tend to increase the antiferment titer—unsaturated fats, phosphorus—have on empiric grounds been employed in the therapy of spasmophilia for many years, indicating that a suppression of a possibly faulty splitting of proteins may form the basis for this theory. Our interest in experimental tetany has centered chiefly, therefore, in a study of the changes of the serum and the relative amounts of split products therein contained.

The experiments were made on dogs. Thyroparathyroidectomy was performed in fifteen animals, and the serum changes were studied. The following two experiments are typical of the relations noted.

*Dog 1.*—Weight 5 kilos. Complete thyroparathyroidectomy on June 1, 1915. Tetany was observed on the 3rd day following the operation; on the 4th day the dog showed no symptoms; but on the next day (June 5, 1915) there was marked tetany. The animal was found dead the following morning. In Text-fig. 1 the serum changes are illustrated in detail.

*Dog 2.*—Weight 8 kilos. Complete thyroparathyroidectomy on June 8, 1915. Tetany was noted in the afternoon of June 10, 1915, again in the following afternoon, and the next morning (June 12, 1915), when the animal was killed. The serum changes are shown in Text-fig. 2.

In the first dog there will be noted a gradual increase in the anti-ferment titer until the time of death, with an irregular protease curve.



TEXT-FIG. 1



TEXT-FIG. 1. Serum changes following thyroparathyroidectomy in Dog 1.

THYROID

PHOSPHATASE  
1.0  
0.9  
0.8  
0.7  
0.6  
0.5  
0.4  
0.3  
0.2  
0.1

ALPASE  
10 CC  
9  
8  
7  
6  
5  
4  
3  
2  
1

PHOSPHATASE  
1.0  
0.9  
0.8  
0.7  
0.6  
0.5  
0.4  
0.3  
0.2  
0.1

AMINO NITROGEN  
PER CC OF SERUM  
1.0  
0.9  
0.8  
0.7  
0.6  
0.5  
0.4  
0.3  
0.2  
0.1

Text-Fig. 2. Serum changes following thyroparathyroidectomy in Dog. 2.

The maximum protease activity was noted in the animal during the time when the tetany was most apparent. The non-coagulable nitrogen of the serum increased to more than twice the original amount. The lipase remained constantly low. The proteoses increased markedly. The amino nitrogen of the serum in this animal showed no change except an initial decrease.

In Experiment 2 (Dog 2) the conditions are different. The anti-ferment titer showed marked fluctuation, the first decline appearing shortly after the operation. The protease remained low until the last day, but the non-coagulable nitrogen increased as in the previous animal; the proteoses also accumulated during the period of tetany. The increase in amino-acids is similar to that observed in practically all the other animals during tetany. This is the only animal of the entire series in which a rise in the lipase titer was observed.

#### DISCUSSION.

It is apparent that the ferment changes in these animals are not in themselves related to the onset of tetany, because the protease and lipase titers, as well as the antiferment index, have no constant relation at any time. It is equally apparent that a considerable accumulation of non-coagulable nitrogen occurs in the serum long before the onset of the actual tetany.

Thus an average of all the dogs examined gives the following figures for the non-coagulable nitrogen per cc. of serum:

Before operation.	24 hrs.	48 hrs.	72 hrs.	96 hrs.
0.21 mg.	0.31 mg.	0.39 mg.	0.50 mg.	0.44 mg.

The average proteose content per 5 cc. of serum was as follows:

Before operation.	24 hrs.	48 hrs.	72 hrs.	96 hrs.
0.41 mg.	0.50 mg.	0.58 mg.	0.74 mg.	0.84 mg.

In four animals of the series the onset and recovery from tetany followed at equal time intervals, and the amino-acid content of the serum per cc. has been averaged under comparable conditions.

Before operation.	24 hrs.	48 hrs.	72 hrs.	96 hrs.
0.48 mg.	0.48 mg.	0.59 mg.	0.54 mg.	0.59 mg.
Operated.	No tetany.	Tetany.	No tetany.	Tetany.

Some observers, among them Morel (6), consider the pathological effect of the parathyroidectomy due to an auto-intoxication, regarding the tetany as a secondary character. An increased nitrogenous metabolism following the removal of the parathyroids is well recognized, but it is difficult to obviate the effect of the tremendous muscular stimulation in the interpretation of the result. It is interesting to note that Falta and Kahn (7) noted an increase in the peptide nitrogen of the urine in their animals, a result possibly of the large increase in the serum which we have observed.

Whether or not the alteration in the nitrogenous constituents indicated by the serum and urine changes is wholly the result of the increased muscular activity due to the increased irritability of the nervous system, or whether the change in these constituents from a quantitative and possibly a qualitative point of view is the cause of the increase of irritability of the nervous system, is, of course, not determined in these experiments.

During the course of the work it was observed that despite the marked fluctuation of the serum protease the titer of the serum lipase remained constantly at a very low level. Stuber and Heim (8) have previously expressed the opinion that the titer of the serum lipase (esterase) was at least partially under the control of the glands of internal secretion. In a series of dogs we have injected dried typhoid bacteria (10 to 20 mg.), which normally cause a prompt and not inconsiderable mobilization of serum lipase, into the circulation of thyroparathyroidectomized dogs and into dogs after complete parathyroid and partial thyroid removal. In the majority of instances the mobilization of lipase has been less constant and of less magnitude than in normal dogs, but the experiments are not sufficiently clear cut to warrant the conclusion of a definite relation between the glandular function and the lipase titer.

## CONCLUSIONS.

1. In thyroparathyroidectomized dogs the onset of tetany bears no constant relation to the ferment-antiferment balance of the serum.
2. The serum lipase titer remains at a low level throughout.
3. A progressive increase in non-coagulable nitrogen and proteoses is observed in the serum following the removal of the glands.
4. The amino nitrogen of the serum is usually increased at the time when tetany is most marked.

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7. Falta, W., and Kahn, F., *Ztschr. f. klin. Med.*, 1912, lxxiv, 108.
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# THE DISAPPEARANCE OF DEXTROSE FROM THE BLOOD AFTER INTRAVENOUS INJECTION.

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## HISTORICAL.

Claude Bernard<sup>1</sup> was the first to study the elimination of sugars after intravenous administration. He, as well as others,<sup>2</sup> demonstrated that dextrose, lactose, or saccharose, when thus injected in large quantity, is eliminated in the urine. Although working only qualitatively, he<sup>3</sup> realized that dextrose was better assimilated under these circumstances than saccharose or lactose. The time relations were worked out, particularly by von Becker, who found after injecting 1.5 gm. of dextrose into rabbits that sugar elimination by the kidneys began promptly and continued for 5 or 6 hours. Limpert and Falck<sup>4</sup> did the first quantitative work. Dogs of 5 or 6 kilos when given 5 to 7 gm. of dextrose intravenously excreted only traces in the urine. Larger amounts led to a slightly increased excretion. Thus it was shown early that after intravenous injection fairly large amounts of dextrose can be retained. The proportion excreted in the urine, according to von Brasol<sup>5</sup> and others<sup>6</sup> bears no constant relation to the injected dextrose; nor can the duration of the glycosuria be predicted. Von Brasol also found that the degree and duration of glycosuria were not always the same for

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<sup>1</sup> Bernard, C., Dissertation, Neue Funktion der Leber als zuckerbereitendes Organ des Menschen und der Thiere, Würzburg, 1853.

<sup>2</sup> Kersting, *Jour. f. prakt. Chem.*, 1844, xxxiii, 58. Baumert, M., *Jour. f. prakt. Chem.*, 1851, liv, 357. Uhle, Inaugural Dissertation, Leipzig, 1852. von Becker, F. J., *Ztschr. f. wissenschaft. Zool.*, 1854, v, 123. All quoted by Limpert, L., and Falck, C. P., *Virchows Arch. f. path. Anat.*, 1856, ix, 56.

<sup>3</sup> Bernard, C., *Compt. rend. Acad.*, 1846, xxii, 536.

<sup>4</sup> Limpert, L., and Falck, C. P., *Virchows Arch. f. path. Anat.*, 1856, ix, 56.

<sup>5</sup> von Brasol, L., *Arch. f. Physiol.*, 1884, 211.

<sup>6</sup> Pavy, F. W., *Jour. Physiol.*, 1899, xxiv, 479. Weyert, F., *Arch. f. Physiol.*, 1891, 187. Lilienfeld, C., *Ztschr. f. diätet. u. physik. Therap.*, 1898-99, ii, 209. Wilenko, G. G., *Arch. f. exper. Path. u. Pharm.*, 1911, lxvi, 143. Bang, I., *Der Blutzucker*, Wiesbaden, 1913, 76.

a given animal. Gilbert and Carnot,<sup>7</sup> however, maintained that within certain limits the proportion of sugar eliminated is constant for each animal, and Blumenthal<sup>8</sup> found that the "assimilation limit;" i.e., the amount which can be injected intravenously without causing glycosuria, is practically invariable for each individual. The velocity of injection plays a part, for Doyon and Dufourt<sup>9</sup> found that more is excreted after rapid than after slow injection. According to them, age, state of nutrition, ligation of the bile duct, and administration of alcohol are apparently without influence. Bang<sup>10</sup> has shown that the glycosuria and glycemia do not run parallel. The concentration of the injected sugar probably plays a part, for Wilenko<sup>11</sup> states that concentrated dextrose solutions (40 per cent) produce the same changes in renal permeability as concentrated salt solutions; that is, first an increased and then a decreased permeability for sugar.

During the injection of dextrose the blood sugar rises and afterwards falls, until it finally assumes a normal or subnormal figure. Von Brasol<sup>5</sup> injected from 0.9 to 5.3 gm. per kilo of body weight in 4 to 6 minutes. Analysis indicated that in the first 2 minutes following the injection some of the sugar had already left the blood; but no direct ratio existed between the amount injected and the blood sugar percentage. After 2 hours the sugar content of the blood was usually normal; with smaller doses (0.9 and 2.3 gm. per kilo) this level was sometimes reached in 1 hour. Butte<sup>12</sup> injected 3 to 10 gm. of dextrose per kilo intravenously and noted the rapid fall of blood sugar to or nearly to the normal level. With 4 gm. per kilo for example, the percentage of sugar in the blood 1½ hours after the injection was 0.353 per cent, and 2 hours after, 0.220 per cent. In no case did he find a subnormal figure as did Grèhant<sup>13</sup> who found 0.036 per cent blood dextrose 2 hours after an injection of 6 gm. of dextrose per kilo. Pavy<sup>14</sup> found in rabbits that the blood sugar fell rapidly after an intravenous injection; for example, immediately after 4 gm. per kilo had been injected, the blood contained 1.4 per cent sugar; 5 minutes after, 1 per cent; and 15 minutes after, 0.8 per cent. Lépine<sup>15</sup> found that after the injection of 1 gm. of dextrose per kilo slowly into dogs, the blood sugar fell in the course of an hour below 0.1 per cent. Bang<sup>16</sup> injected 1 to 2 gm. per kilo intravenously and found that the maximum blood sugar was reached 2 to 5 minutes after the injection and that the high percentage was maintained for about 30 minutes, after which a rapid fall to normal or below

<sup>7</sup> Gilbert and Carnot, *Compt. rend. Soc. de biol.*, 1898, v, 330.

<sup>8</sup> Blumenthal, F., *Beitr. z. chem. Phys. u. Path.*, 1905, vi, 329.

<sup>9</sup> Doyon, M., and Dufourt, E., *Jour. de physiol. et de path. gén.*, 1901, iii, 703.

<sup>10</sup> Bang, I., *Der Blutzucker*, Wiesbaden, 1913, 77.

<sup>11</sup> Wilenko, G. G., *Arch. f. exper. Path. u. Pharm.*, 1911, lxxvi, 143.

<sup>12</sup> Butte, L., *Compt. rend. Soc. de biol.*, 1896, iii, 274.

<sup>13</sup> Grèhant, quoted by Butte, L., *Compt. rend. Soc. de biol.*, 1896, iii, 274.

<sup>14</sup> Pavy, F. W., *Jour. Physiol.*, 1899, xxiv, 479.

<sup>15</sup> Lépine, R., *Le diabète sucré*, Paris, 1909, 200.

<sup>16</sup> Bang, I., *Der Blutzucker*, Wiesbaden, 1913, 74.



took place. Using animals with ligated ureters, Weyert<sup>17</sup> found that after the injection of 4.4 to 5.8 gm. of dextrose per kilo, the blood sugar was down to nearly normal limits in 3 hours, and Harley<sup>18</sup> found that with a dosage of 10 gm. per kilo, the blood sugar fell to normal in from 3 to 6 hours.

Weyert<sup>17</sup> records that the sugar concentration of the lymph runs close to that of the blood. He also found traces of sugar in the cerebrospinal fluid and in the vitreous humor of the eye. None can be found in the saliva except after very large doses,<sup>9, 17, 19</sup> nor is an appreciable amount secreted by the intestine<sup>9, 20, 21</sup> or by the wall of the urinary bladder.<sup>22</sup>

The possibility of the conversion of injected dextrose into glycogen must, of course, be considered. Voit<sup>23</sup> injected subcutaneously 50 gm. of dextrose into three rabbits and found in the liver only 1.4, 2.2, and 7.0 gm. of glycogen, respectively, showing that sugar is not as readily converted into glycogen by the liver when injected parenterally as when fed. Harley<sup>18</sup> after injecting 10 gm. of dextrose per kilo intravenously into dogs with the ureters tied, found some slight evidence of glycogen formation. Freund and Popper<sup>24</sup> analyzed a lobe of liver before injecting dextrose intravenously and the rest of the liver afterwards, and found a small increase in liver glycogen, which was greater if the animals had previously been starved for a short time. With starved dogs 4 gm. per kilo gave no increase in glycogen, but 7 to 11 gm. per kilo resulted in the formation of 1.3 to 7 gm. of glycogen.

Inasmuch as the injected sugar is accounted for only in small part by glycogen and by the various secretions, the blood at the same time rapidly assuming its normal content of sugar, the question naturally arises: What is the fate of the rest of the sugar? In seeking to answer this question, various facts have been brought out. Von Brasol<sup>5</sup> injected 12 to 18 gm. of dextrose per kilo intravenously into rabbits in 30 to 45 minutes and then analyzed the blood, the urine, and mixed samples of muscle, kidney, and liver. Estimating the relation of blood to body weight even as high as 12 per cent and even assuming that all the tissues, including bones, hair, etc., contained the same proportion of dextrose as the tissues analyzed, he calculated that from 17.5 to 28.7 per cent of the sugar administered, was still to be accounted for. Von Brasol did not determine glycogen or other substances; in fact he suggests that the missing fraction has been converted into glycogen, or lactic acid or some other substance. Butte<sup>13</sup> made muscle analyses after injecting intravenously 4 gm. of dextrose per kilo into a dog. Assuming

<sup>17</sup> Weyert, F., *Arch. f. Physiol.*, 1891, 187.

<sup>18</sup> Harley, V., *Arch. f. Physiol.*, 1893, Supplement, 46.

<sup>19</sup> Jappelli, A., *Ztschr. f. Biol.*, 1908, li, 435.

<sup>20</sup> Bang, I., *Der Blutzucker*, Wiesbaden, 1913, 80.

<sup>21</sup> Kleiner, I. S., *Jour. Exper. Med.*, 1911, xiv, 274.

<sup>22</sup> Kleiner, I. S., *Jour. Exper. Med.*, 1913, xviii, 310.

<sup>23</sup> Voit, C., *Ztschr. f. Biol.*, 1891, xxviii, 245.

<sup>24</sup> Freund, E., and Popper, H., *Biochem. Ztschr.*, 1912, xli, 56.

that the muscle contained no sugar before the injection, he noted a rise to 0.42 per cent half an hour after the injection and then a progressive fall in the percentage of dextrose. This demonstrates, according to Butte, that one part of the injected sugar is transformed in certain organs, although he does not mention the likelihood of bacterial action in a dead animal. In two experiments on rabbits Bang<sup>20</sup> analyzed the liver, skin, blood and lymph, kidneys and urine, muscles, intestines, and bones for free dextrose after injecting 4 gm. intravenously into each animal. The total amount recovered was 47.9 and 75.5 per cent, respectively. But, according to Bang, even these figures are too high because (a) preformed sugar was not determined, (b) the tissues were not blood-free and hence blood sugar was counted twice, and (c) all reduction was not necessarily due to sugar.

The rapid transformation of dextrose into simpler substances, particularly lactic acid, has been suggested by many investigators. Apparently the only one who actually tested for lactic acid after intravenous injections of dextrose was Harley.<sup>18</sup> He found a higher percentage of lactic acid in the blood after dextrose injections into animals with the ureters tied, and also an increase in this substance in the liver and muscle. He also obtained qualitative tests for ethyl alcohol and acetone in the blood. Although some workers have brought forth evidence in favor of a protein origin for lactic acid, there seems to be no doubt that it can be formed from sugar. For example, Embden and his collaborators<sup>25</sup> by perfusing livers with blood found that the liver could form lactic acid from glycogen in the liver or dextrose in the blood. More recently Levene and Meyer<sup>26</sup> have observed a direct conversion of sugar into lactic acid by leukocytes.

An increase in the respiratory quotient after intravenous dextrose injections has been observed by Harley,<sup>27</sup> Verzár and Fejér,<sup>28</sup> and others.

The following points appear, therefore, to be established: (1) The intravenous injection of large quantities of dextrose is followed by an increase in the percentage of sugar in the blood. (2) The high content of sugar in the blood falls rapidly after the end of the injection and assumes the normal value in a comparatively short time, even when the ureters are tied. (3) The kidneys eliminate a considerable, but variable, fraction of the injected sugar. (4) No sugar, or mere traces are to be found in the cerebrospinal fluid, saliva, intestinal secretions, or in the secretion of the mucosa of the urinary bladder. (5) An increase of glycogen in the liver occurs with very large doses, but it accounts for only a small fraction of the sugar injected. (6) Tissue analyses, although few in number, show that some of the sugar is to be found unaltered in various organs. (7) Some of the sugar is oxidized, or at least the respiratory quotient is increased.

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<sup>25</sup> Embden, G., *Centralbl. f. Physiol.*, 1904-05, xviii, 832. Embden, G., and Kraus, F., *Biochem. Ztschr.*, 1912, xlv, 1.

<sup>26</sup> Levene, P. A., and Meyer, G. M., *Jour. Biol. Chem.*, 1912, xi, 361.

<sup>27</sup> Harley, V., *Jour. Physiol.*, 1894, xv, 139.

<sup>28</sup> Verzár, F., and von Fejér, A., *Biochem. Ztschr.*, 1913, liii, 146.

The chief purpose of the present work was to study the disappearance of sugar from the blood under various conditions. In order to obtain a basis of comparison a series of experiments was first carried out in which dextrose was injected into normal animals.

#### EXPERIMENTAL PART.<sup>29</sup>

The experiments were performed on dogs. Ether was administered by intratracheal insufflation, and cannulas were inserted in the left jugular vein, the right carotid artery, and the neck of the urinary bladder. In a few of the later experiments only morphine and cocaine were employed as anesthetics. In the experiments in which the kidneys were ligated, they were reached through the lumbar region. At the completion of all operative work, the animal was given morphine, usually 0.01 gm. per kilo, subcutaneously, and then the insufflation and ether anesthesia were discontinued. After 1½ to 2½ hours a sample of blood was taken from the artery, and a warm solution of 20 per cent dextrose was injected slowly into the jugular vein. The rate of injection was about 2.5 to 3 cc. per minute and the dosage was 4 gm. per kilo of body weight. At the middle and end of the injection and at intervals thereafter, samples of blood (from 10 to 30 gm.) were taken for analysis. Urine elimination was divided into periods corresponding to the blood samples taken. During the experiment the animal was kept on an electric thermal pad and at the end of the experiment it was chloroformed.

The blood was analyzed for dextrose by removing the protein by Reid's method,<sup>30</sup> and the reduction was determined by means of a Pavy<sup>31</sup> solution. The urine

<sup>29</sup> A preliminary report of this work was published by Kleiner, I. S., and Meltzer, S. J., *Am. Jour. Physiol.*, 1914, xxxiii, p. xvii.

<sup>30</sup> Reid, E. W., *Jour. Physiol.*, 1896, xx, 316. Vosburgh, C. H., and Richards, A. N., *Am. Jour. Physiol.*, 1903, ix, 35. Macleod, J. J. R., *Jour. Biol. Chem.*, 1908-09, v, 443.

<sup>31</sup> The reagent used was modified from time to time. Vernon (*Jour. Physiol.* 1902, xxviii, 156) suggested the use of twice as much Rochelle salt, potassium hydroxide, and ammonium hydroxide as Pavy originally employed. As this results in the formation of a heavy crystalline deposit in the reagent on standing, we have decreased the amount of Rochelle salt and potassium hydroxide. The resulting solution, which deposits very little precipitate, gives good results. The composition of the solution, as we now prepare it, is:

CuSO <sub>4</sub>	4.158 gm.
Rochelle salt	14 "
KOH	17 "
NH <sub>4</sub> OH (sp. gr. 0.88)	600 cc.
H <sub>2</sub> O to	1,000 "

10 cc. of this solution corresponds to 0.005 gm. dextrose, but one should determine the exact equivalent every time the reagent is made up, and this should be checked at intervals.

was also analyzed by the Pavy method. Although this method is not as accurate as some of the other sugar methods, its rapidity, and the fact that a number of determinations can be made from a small quantity of a given solution (as each test requires only 5 mg. of dextrose), led us to use it in this investigation. In some experiments, however, the Bertrand method was used to determine the reducing power of the blood after removal of the proteins, and in a few of the experiments we have used the blood sugar method of Lewis and Benedict as modified by Myers and Bailey,<sup>22</sup> which we have recently adopted for use in this laboratory. With this method only 2 cc. of blood is required.

### *Normal Animals.*

The first dextrose injections were given to seven normal animals. The following is a typical protocol.

*Experiment LD 5.*—White bull terrier, female; weight 6.5 kilos.

11.00. Etherized. Intratracheal insufflation begun and ether anesthesia continued by this method. Cannulas now inserted in the left jugular vein, left carotid artery, and in the neck of the bladder.

11.51. Urine obtained from bladder; 1.69 per cent dextrose.

11.56. Ether discontinued.

12.02. 6.5 cc. 1 per cent morphine sulphate injected subcutaneously.

12.13. Insufflation discontinued.

2.07. Blood taken, 22.3 gm.; 0.28 per cent dextrose.

2.09. Urine, 77 cc. + (about 5 cc. lost), 8.84 per cent = 6.81 gm. dextrose.

2.10. Injection of warm 20 per cent dextrose solution into jugular vein started.

2.34. Blood taken, 9.5 gm., 0.68 per cent (67 cc. of dextrose have been injected).

2.36. Urine, 40 cc., 6.84 per cent = 2.74 gm. (70 cc. of dextrose have been injected).

2.56. Dextrose injection ends. Total amount injected 130 cc., or 26 gm. in 46 minutes.

2.57. Blood taken, 10.0 gm., 0.93 per cent.

2.58. Urine, 81 cc., 5.50 per cent = 4.46 gm.

3.27. Blood taken, 16.6 gm., 0.40 per cent.

3.28. Urine, 51 cc., 7.32 per cent = 3.73 gm.

3.57. Blood taken, 17.9 gm., 0.30 per cent.

3.58. Urine, 11.5 cc., 10.6 per cent = 1.22 gm.

4.27. Blood taken, 21.3 gm., 0.28 per cent.

4.29. Urine, 3.2 cc., 8.59 per cent = 0.28 gm.

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<sup>22</sup> Lewis, R. C., and Benedict, S. R., *Jour. Biol. Chem.*, 1915, **xx**, 61. Myers, V. C., and Bailey, C. V., *Post-Graduate*, 1915, **xxx**, 31; *Jour. Biol. Chem.*, 1916, **xxiv**, 147.

*Summary.*—1½ hours after the end of the dextrose injection the blood sugar fell to its original level of 0.28 per cent.

Dextrose injected.....	gm. 26.0
Excess dextrose (above original value) in circulating blood at end of experiment.....	0.00
Dextrose contained in blood samples taken for analysis.....	0.13
Dextrose in urine.....	12.43
Dextrose accounted for.....	12.56
(or 48.5 per cent of the amount injected)	
Dextrose not accounted for.....	13.44
(or 51.5 per cent of the amount injected)	

The results of the seven experiments are given in Table I.

Considering first the glycemia, it is seen that in five out of seven experiments, the original blood sugar was high. This was to be expected, as the animals had been subjected to being tied down, to anesthesia (ether and morphine), and to operative procedure, all of which are known to raise the blood sugar.<sup>23</sup> An initial rapid rise in the blood sugar during the injection is indicated in each case by the figures for the beginning and middle of the injection. Then a more gradual rise occurred until the end of the injection, after which the percentage of blood sugar fell rapidly for half an hour and then more slowly. At the end of 1½ hours the percentage of sugar in the blood fell to its original level in three experiments (Experiments 3, 5, and 46) and nearly to its original level in two others (Experiments 6 and 7). In the two remaining experiments (4 and 47) the last figures were twice as great as the first. However, in one of these two (Experiment 47) the last percentage found was, nevertheless, a low figure when compared with the rest of the series. In other words, in only one of the seven experiments did the blood sugar fail to fall to what might be termed a low figure for this series.

In Experiments LD 4, 5, and 6, and LP 46, the urine secreted during the injection contained more sugar than that secreted during the 90 minute after-period. This was due to a greater volume of urine being secreted at first rather than a higher percentage of sugar.

<sup>23</sup> For a discussion of these points see Shaffer, P. A., *Jour. Biol. Chem.*, 1914, xix, 297. Loewy, A., and Rosenberg, S., *Biochem. Ztschr.*, 1913, lvi, 114. Hirsch, E., and Reinbach, H., *Ztschr. f. physiol. Chem.*, 1914, xci, 292.

**TABLE I.**  
*Intravenous Injection of Dextrose into Normal Dogs.*

[illegible]

**\* Only morphine anesthesia.**

† Average of five.

† Average of six.

**§ Average of seven.**

Indeed, the urine of the after-period was usually of a higher concentration, and in Experiment LP 47 this was sufficient to cause a greater excretion of sugar in the after-period than in the injection period.

The small flow of urine in Experiment LD 3 was probably due to over-etherization. Excluding this experiment, the average amount found in the urine was 60.2 per cent of the sugar injected. If we now estimate the excess sugar still circulating in the blood (calculating the blood equal to 7 per cent of the body weight) and add this blood sugar to the urinary sugar, we have an average of 62.2 per cent of the injected amount, leaving 37.8 per cent not accounted for. If we include Experiment LD 3, this average is raised to 46.3 per cent not accounted for.

It thus appears from our own experiments on normal animals that approximately 60.2 per cent of the sugar was excreted by the kidneys, while of the remaining 39.8 per cent only an insignificant fraction (2 per cent on an average) remained in the circulation.

#### *Nephrectomized Animals.*

In one of our experiments on normal animals (Experiment LD 3) there occurred, as mentioned above, an almost complete suppression of the kidney function during and after the intravenous infusion of dextrose. Nevertheless, the blood sugar returned to its original value as quickly as in the other experiments. We now performed some experiments upon animals after ligating or removing the kidneys. The results of five experiments are summarized in Table II.

The following is a typical protocol.

*Experiment LD 33.*—Fox-terrier, male; weight 6.75 kilos.

10.18. Etherized. Intratracheal insufflation begun and ether anesthesia continued by this method. The kidneys were now exposed and ligated and cannulas inserted in left jugular vein and right carotid artery.

11.15. Ether discontinued.

11.26. Lid reflex present.

11.27. Blood taken, 11.6 gm., 0.28 per cent.

11.31. Insufflation discontinued.

11.33. 1 cc. 1 per cent morphine sulphate injected intramuscularly.

12.27. Blood taken, 11.35 gm., 0.27 per cent.

12.28. Injection of warm 20 per cent dextrose solution into jugular vein started.

1.22. Dextrose injection ended. Total amount introduced, 135 cc. or 27 gm. in 54 minutes.

1.23. Blood taken, 11.9 gm., 1.08 per cent.

1.29. 0.5 cc. 1 per cent morphine sulphate injected intramuscularly.

1.54. Blood taken, 11.5 gm., 0.69 per cent.

2.24. Blood taken, 14.9 gm., 0.40 per cent.

2.54. Blood taken, 15.95 gm., 0.32 per cent.

*Summary.*—1½ hours after the end of the injection the blood sugar fell to 0.32 per cent; *i.e.*, only 0.05 above its original level of 0.27 per cent.

Dextrose injected.....	gm.	27.0
Excess dextrose (above original value) in circulating blood at end of experiment .....	about	0.27
Dextrose contained in blood samples taken for analysis ....	about	0.17
Total dextrose thus accounted for.....		0.44
(or 1.7 per cent of the amount injected)		
Dextrose not accounted for.....		26.56
(or 98.3 per cent of the amount injected)		

TABLE II.

*Intravenous Injection of Dextrose into Nephrectomized Dogs.*

No. of experiment.	Dextrose injected (4 gm. per kilo).	Blood sugar before injection.		Blood sugar at end of injection.	Blood sugar ½ hr. after injection.	Blood sugar 1 hr. after injection.	Blood sugar 1½ hrs. after injection.	Blood sugar 2 hrs. after injection.	Sugar not accounted for. Per cent of total amount injected.
	gm.	cc.	per cent	per cent	per cent	per cent	per cent	per cent	per cent
LD 32 .....	11.0	55		0.47	1.09	0.93	0.70		100
LD 33 .....	27.0	135	0.28	0.27	1.08	0.69	0.40	0.32	98.3
LD 34 .....	42.0	210	0.30	0.35	1.0		0.55	0.46	99.6
LP 48* .....	43.4	217		0.10	0.62			0.13	99.5
LP 49* .....	37.0	185		0.28	1.03			0.27	100
Average .....				0.29	0.96			0.295	99.5

\* In these two experiments the kidneys were exposed and prepared for ligation under ether anesthesia, but they were not actually ligated until the effects of the ether had worn off.



In these animals the blood sugar rises to a higher level, but this is not as great as might be expected. In the normal dogs the average figures were 0.19 before injection and 0.77 at the end, a rise of 0.58 per cent; in the nephrectomized animals the corresponding figures are 0.29 and 0.96, a rise of 0.67 per cent; hence there is a difference between the two series of only 0.09 per cent. If we now estimate from this figure how much more sugar there is circulating in the blood in this series, we find that it amounts to less than 1 gm. Therefore during the injection the organism without the kidney has been enabled to rid its blood of nearly as much sugar as if it had its renal function intact. After the discontinuation of the injection the fall in the glycemia is most rapid during the first hour and then becomes slower. In Experiments LD 33, LD 34, LP 48, and LP 49 the blood sugar is either at its original level or only slightly higher at the end of  $1\frac{1}{2}$  hours, and in Experiments LD 32 and 34 the figures for 2 hours show that by this time the blood sugar has fallen to or below this value. In Experiment 32 no sample was taken at the end of  $1\frac{1}{2}$  hours, but the average for the other four experiments for this time was 0.30 per cent, while the average before injection was 0.25 per cent in the same four.

It is thus evident that after an intravenous injection of dextrose the blood sugar does not rise much higher in nephrectomized animals than in normal animals, and after injection gradually falls to or nearly to its original level. This indicates, of course, that practically all the injected sugar has left the blood stream; and that therefore the body is able to dispose of large amounts of injected dextrose even without the assistance of the kidneys. Furthermore, the fact that this was accomplished in the same length of time as in the normal series, the dosage and other experimental conditions remaining the same, shows that the presence or absence of the kidney has very little influence on the rate of disappearance of dextrose from the blood.

The obvious explanation which offers itself at first thought is that the greater part of the injected sugar is rapidly converted into glycogen by the liver. We have performed some experiments which were designed to exclude this possibility. Dextrose was injected into dogs in which there was practically no circulation posterior to the diaphragm. Animals can easily be prepared in this manner since, with

the aid of intratracheal insufflation, the thorax can be opened and the aorta and vena cava inferior tied near the diaphragm with great facility.

*Animals with a Circulation only Anterior to the Diaphragm.*

The animals were prepared as follows: An opening was made in the left side of the thorax (under intratracheal insufflation anesthesia) and the aorta and vena cava were ligated as near the diaphragm as possible. The aorta was ligated first, and before ligating the vena cava, pressure was applied to the abdomen in order to increase the amount of blood in the thorax. In some cases Ringer's solution or saline, in others adrenalin, or both, were injected to maintain the blood pressure. These animals, then, had no abdominal circulation; the liver could store no glycogen; the kidneys, pancreas, and adrenals, could not function. Into such animals, we injected dextrose as before and estimated the glycemia at intervals. Some experiments were complicated by such variations as removal of the thyroids, tying the thoracic duct, etc. These additional factors exerted no appreciable influence on the results, and we shall not deal with them in particular. Some of the experiments are summarized in Table III.

A typical experiment of this series is the following:

*Experiment LD 30.*—Dog; weight 7.5 kilos.

1.40. Etherized. Intratracheal insufflation begun and ether anesthesia continued by this method. Cannulas now inserted in left jugular vein and right carotid artery. Thoracic duct exposed. Opening made in left side of thorax and aorta tied near diaphragm. After pressure on the abdomen the vena cava inferior was tied near diaphragm.

2.43. Wound in thorax closed.

2.44. Ether discontinued.

2.46. Thoracic duct tied.

2.47. Blood taken, 10.9 gm., 0.07 per cent.

2.48–2.50. 40 cc. warm sterile Ringer's solution injected in left jugular vein.

3.04–3.05. 20 cc. Ringer's solution injected.

3.18. 10 cc. Ringer's solution injected.

3.23. Pulse poor. Dog restless. 20 cc. Ringer's solution injected.

3.31–3.32. 20 cc. Ringer's solution injected. (Total quantity Ringer's solution injected, 110 cc. during 44 minutes).

3.40. 1 cc. 1 per cent morphine injected intramuscularly in left fore-leg.

3.49. Blood taken, 10.8 gm., 0.05 per cent.

3.50. Injection of warm 20 per cent dextrose in left jugular vein started.

4.30. Dextrose injection ended. Total amount injected, 150 cc. 20 per cent dextrose or 30 gm., in 40 minutes.

4.32. Blood taken, 14.7 gm., 1.65 per cent.

4.48. Pulse good.

5.27. Blood taken, 22.6 gm., 1.16 per cent.

*Summary.*—Estimated weight of anterior part of animal; *i.e.*,

one half of total body weight..... 3,750 gm.

Quantity of blood circulating (7 per cent of weight)..... 262 cc.

Fluid injected..... 260 "

522 "

Blood samples taken for analysis.....about 39 "

Total circulating fluid.....about 483 "

Excess dextrose circulating  $((1.16-0.05 \text{ per cent}) \times 483 \text{ cc.})$  5.36 gm.

Dextrose in blood samples taken..... 0.26 "

Total dextrose accounted for.....about 5.62 "

Dextrose injected..... 30 "

Amount of dextrose not accounted for, 24.4 gm. or 81 per cent of the quantity injected.

The interpretation of these figures is made difficult by several complications. As we injected the same amount of dextrose (4 gm. per kilo) as in the first two series, the dosage of dextrose per kilo was manifestly higher in these experiments since the posterior part of the body was not fed by the circulation. Exactly how much higher cannot be said definitely, but probably the remaining circulation received about twice as much dextrose per kilo as in the experiments on the entire animal. The infusion of Ringer's solution and the occasional use of adrenalin also make these experiments not entirely comparable with the others. However, the first blood sample was always taken after injection of Ringer's solution or adrenalin, so that a comparable initial glycemia was always ascertained.

In these experiments the blood sugar rose greatly; at the end of the injection it reached from 1.35 to 2.41 per cent, an average of 1.93 per cent for eleven experiments. In considering these figures it must be remembered that, as stated above, the dose of sugar per volume of circulating blood was about twice as high as in the other two series. In every experiment there was a rapid fall in the blood sugar after the injection had been finished. Since the samples of blood were not always taken at the same intervals, it becomes necessary to study the experiments in groups. In Experiments 55, 56, and 57 the

TABLE III.

*Intravenous Injection of Dextrose into Dogs with Aorta and Vena Cava Ligated near the Diaphragm.*

No. of experiment.	Dextrose injected (4 gm. per kilo).		Blood sugar before injection.	Blood sugar at end of injection.	Blood sugar 1/4 hr. after in- jection.	Blood sugar 1 hr. after in- jection.	Blood sugar 1 1/2 hrs. after in- jection.	Blood sugar 2 hrs. after in- jection.	Muscle before injection.		Muscle after injection.		Sugar not accounted for. Per cent of total amount injected.	
	gm.	cc.	per cent	per cent	per cent	per cent	per cent	per cent	Dex- trose.	Polysac- char- ides.	Dex- trose.	Polysac- char- ides.	By blood.	By blood and muscle. per cent
LD18.....	18.0	90		2.33		1.44							82	
LD20.....	25.6	128	0.15 (3 hrs. later)	2.03	1.16								87	
LD21* †.....	28.0	140	0.14 (1 hr. later)	2.34	1.55								80	
LD28* §.....	41.0	205	0.19	2.18	1.58								78	
LD30*   .....	30.0	150	0.07 (1 hr. later)	1.65	1.16								82	
LD31† ¶.....	41.0	205	0.10	1.65	1.18		1.13						83	
LD35† *.....	27.0	135	0.40	1.81	1.34								86 (1 hr.) 89 (2 hrs.)	
LD45* **.....	34.0	170		1.75					0.26	0.15	0.49	0.21	74	
LD55.....	37.0	176	0.33	1.35	1.02				0.40	0.18	0.36	0.24	91	82
LD56*.....	29.8	144	0.15	2.41	1.70				0.52	0.54	0.71	0.50	81	49
LD57*.....	36.7	183.7	0.17	1.73	1.38						0.80	0.37	91	86
Average.....				1.93†† 1.83†† 2.00§§	1.37 1.34									

- \* Thoracic duct ligated.  
† Thoracic duct ligated and thyroid glands removed.  
‡ 15 cc. of Ringer's solution injected between first and second blood samples.  
§ 100 cc. of Ringer's solution and 1 cc. of adrenalin (in small portions) injected before taking first blood sample.  
|| 110 cc. of Ringer's solution (in small portions) injected between first and second blood samples.  
¶ 75 cc. of Ringer's solution injected before taking first blood sample.  
\*\* Ether and magnesium sulphate anesthesia. 40 cc. of Ringer's solution injected before taking first blood sample.  
\*\*\* 60 cc. of Ringer's solution and 0.5 cc. of adrenalin injected before dextrose injection.  
†† Average of eleven.  
‡‡ Average of last three.  
§§ Average of first seven.

TABLE IV.  
*Intravenous Injection of Dextrose into Whole Dead Animals.*

No. of experiment.	Interval between heart stoppage and injection.	Dextrose injected (4 gm. per kilo).		Blood sugar before injection.		After injection.						Muscle before injection.		Muscle after injection.			Dextrose not accounted for. Per cent of total amount injected.	
		gm.	cc.	per cent	Time.	Blood sugar.	Time.	Blood sugar.	Time.	Blood sugar.	per cent	Dextrose.	per cent	Time.	Dextrose.	per cent	By blood.	By blood and muscle.
LD48.....	3	21	105		9	1.95											56	
LD49.....	21	30	150		30	3.26	73	3.85†				0.54	0.15	83	0.88	0.83	30	0
					46	2.83*												
LD51.....	25	22	110		30	1.54						0.29	0.14	42	0.60	0.21	65	27
LD53.....	14	18	90	0.02 (?)	37	1.67	67	1.48	157	1.30(1.01)‡		0.33	0.21	161	0.45	0.22	71	62
LD54.....	10	32	160	0.23	30	2.18	60	2.03	105	1.93		0.38	0.30	126	0.64	0.60	64	6

\* This sample of blood was obtained from the aorta after tying the inferior and superior venæ cavæ.

† This sample of blood was obtained from the portal circulation.

‡ 100 cc. saline were injected in order to get this sample and the figure 1.30 per cent was calculated on the assumption that the saline and blood were intimately mixed.

average percentage of sugar in the blood at the end of the injection was 1.83 per cent, and a half hour later the average was 1.37 per cent—a fall of 0.46 per cent in half an hour. In Experiments 18, 20, 21, 28, 30, 31, and 35, the blood sugar fell from an average of 2.00 per cent at the end of the injection to 1.34 per cent in an hour; in Experiment 57 the fall continued steadily from 1.73 to 1.38 in half an hour, and to 0.82 per cent in  $1\frac{1}{2}$  hours. In Experiment 35 a drop in the blood sugar from 1.81 to 1.34 per cent occurred in the first hour after the end of the injection, while during the second hour only a relatively slight decrease took place; namely, to 1.13 per cent.

In every experiment a large percentage of the injected sugar was not accounted for by the sugar present in the circulating blood, from 74 to 91 per cent disappearing from the blood stream during the time of observation. Another way of demonstrating this is by estimating the percentage of sugar which would have been present in the blood if all the injected sugar was still there and comparing this figure with the actual findings, taking into account the volume of blood drawn and of fluid injected. Such estimations indicate that the blood sugar content would be about 7.7 to 7.9 per cent if all the sugar was in the circulating blood. As a matter of fact the highest blood sugar values in all these experiments ranged from 1.35 to 2.41 per cent, or an average of only 1.93 per cent. It is thus evident that the greater part of the injected sugar left the blood rapidly, in fact before the end of the injection. This disappearance of dextrose from the blood stream went on, then, entirely independently of the abdominal viscera. Hence neither the glycogenic activity of the liver nor an excessive consumption of sugar by or in any abdominal organ can account for the greater part of the dextrose which so rapidly leaves the blood after intravenous infusion.

*Analysis of Muscle for Carbohydrates.*—In a few of these experiments we analyzed skeletal muscles to determine whether sugar had been stored in them. Higher carbohydrates, as well as dextrose were estimated. A few preliminary studies convinced us that the carbohydrate content of normal muscle is not constant. Therefore it was necessary to determine in each experiment the amount of dextrose and higher carbohydrate present in the muscles before and after the injection.

In Experiments 55, 56, and 57 the procedure was as follows: One fore-leg was amputated before the injection of dextrose and immediately placed in the refrigerator. At the end of the experiment the animal was killed, the other fore-leg removed and also placed in the refrigerator. The muscles were dissected off, put through the grinder twice, and extracted with water in the presence of toluene for about 18 hours in the refrigerator. The fluids were then pressed out and the residue again extracted with water for about 2 hours. All fluids obtained were immediately boiled after adding a little acetic acid and filtered through glass wool. The residue, pressing cloth, etc., were thoroughly extracted with boiling water. The combined fluids were then treated with phosphotungstic acid solution while boiling, filtered, neutralized with sodium hydroxide, acidified with acetic acid, and finally concentrated to a definite volume on the water bath. Estimations of the reducing power were made before and after hydrolysis and the results expressed as dextrose. Hydrolysis was effected by boiling under a reflux condenser for  $1\frac{1}{2}$  hours in the presence of 1.8 per cent hydrochloric acid.

In all three experiments there was an increase in the total carbohydrates (*i.e.*, the amount found after hydrolysis) after the injection: There was, however, some slight difference with regard to the behavior of the two types of carbohydrates. In Experiments 55 and 56 the increase in dextrose is practically the same as that of the higher carbohydrates; namely, 0.10 per cent for dextrose and 0.09 per cent for higher carbohydrates in Experiment 55; and 0.31 per cent for dextrose and 0.32 per cent for higher carbohydrates in Experiment 56. In both experiments the time was half an hour. In Experiment 57, where the time was  $1\frac{1}{2}$  hours, there was an increase of 0.28 per cent in the dextrose and a decrease of 0.17 per cent in the higher carbohydrates. The fact that the higher carbohydrates were found to be increased after 30 minutes and decreased after 90 minutes might indicate that the higher carbohydrates were more readily utilized than the lower ones, or that dextrose before being oxidized by the muscle must be converted into a polysaccharide. Our evidence, however, comes from only three experiments, and consequently we cannot attach much importance to it.

Does this increase in the carbohydrate content of muscle account for all the injected dextrose which disappeared from the circulation? Consideration of the following figures will show that we are not entitled to answer this in the affirmative. If we assume that the muscle tissue of the dog constitutes approximately 43 per cent of

the body weight, as in man,<sup>24</sup> and that half the body is reached by the circulating blood, we can estimate roughly the increase in carbohydrates of the muscles. Such calculations show only comparatively small percentages of the injected dextrose. Thus in Experiment LD 55 the amount not accounted for is reduced from 91 to 82 per cent by taking the muscle carbohydrates into consideration; in Experiment LD 57 the reduction is from 91 to 86 per cent; and in Experiment LD 56 it is somewhat greater, namely, from 81 to 49 per cent. From this it seems evident that the anterior muscles in these experiments did not contain all the missing sugar. On the other hand, it must be admitted that one cannot draw definite conclusions from the muscle analyses, for which great accuracy cannot be claimed. Furthermore it is possible that, in these "anterior" animals, loss of dextrose may occur through the tissue spaces into the posterior part of the body, which we have not included in our estimations. Finally we have not examined other tissues, *e.g.*, the brain, lungs, spinal fluid, bone marrow, etc., where the carbohydrates might also have increased in amount.

Thus, while we cannot claim that the experiments on the muscles in anterior animals throw definite light upon the fate of all the dextrose which disappears from the circulation after an intravenous injection, the fact remains that a notable increase in the carbohydrate content of the muscles was found.

*Experiments with Intravenous Injections of Dextrose into Whole Dead Animals.*

The foregoing experiments brought up the question whether this passage of sugar from the blood into the tissues is a vital process; this led to a series of experiments in which dextrose was injected into dead animals.

*Method.*—The dogs were prepared as in the experiments in Tables I and III on normal and anterior animals, except that, under light chloroform insufflation anesthesia, three or four ribs were resected on the left side in order to expose the

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<sup>24</sup> Vierordt, H., *Anatomische physiologische und physikalische Daten und Tabellen*, 3rd edition, Jena, 1906, 44.



heart. Then full chloroform vapor was given until the heart stopped. In some cases one fore-leg was then removed for analysis, all bleeding points being carefully ligated. Continuing the artificial respiration and massaging the heart by hand, we injected the dextrose solution intravenously, much more quickly, however, than in the experiments upon living animals. The rhythmic heart massage was continued for 5 or 10 minutes, and blood samples were taken from the carotid while massaging the heart.

The following is a protocol of one experiment.

*Experiment LD 54.*—Dog, male; weight 8 kilos.

10.35. Etherized. Intratracheal insufflation begun and chloroform anesthesia produced by this method. Cannulas were inserted in the left jugular vein and right carotid artery. The thorax was opened on the left side, four ribs being resected and the heart exposed. The left fore-leg was then amputated.

12.08. Left fore-leg placed in refrigerator.

12.10. Full chloroform vapor given.

12.20. The fibrillary twitchings of the heart have ceased.

12.23–12.24. Blood taken from the carotid artery, 23.03 gm., 0.23 per cent.

12.30–12.32½. 160 cc. of warm 20 per cent dextrose solution injected into left jugular vein, followed by 3 cc. 0.9 per cent sodium chloride. Heart massaged during the injection.

12.32½–12.36. Massaging of heart continued.

1.02–1.04. Blood taken from carotid, 20.04 gm., 2.18 per cent.

1.31–1.34. Blood taken from carotid, 16.07 gm., 2.03 per cent.

2.17–2.18. Blood taken from carotid, 20.74 gm., 1.93 per cent.

2.39. Right fore-leg removed and put in refrigerator.

*Analysis of Muscle of Fore-Legs.*

Control muscle: 0.38 per cent before hydrolysis (dextrose).

0.68 per cent after hydrolysis (total carbohydrates),

or 0.30 per cent polysaccharides.

Muscle after dextrose injection: 0.64 per cent before hydrolysis (dextrose).

1.24 per cent after hydrolysis (total carbohydrates),

or 0.60 per cent polysaccharides.

Increase in total carbohydrates, 0.56 per cent.

*Summary.*—Amount of dextrose injected, 32 gm. 1½ hours after the end of the injection, the blood sugar was 1.93, or 1.70 per cent above its original value. There was estimated to be about 636 cc. of blood in the blood vessels at the time of taking the last blood sample.

Increase in dextrose in blood ( $636 \times 1.70$  per cent) . . . . 10.8 gm.

Dextrose removed in blood samples . . . . . 0.7 “

Total excess dextrose in blood . . . . . 11.5 “

or 36 per cent of amount injected.

Therefore 64 per cent not accounted for by blood.

The amount of muscle was estimated to be 3,300 gm.; that is, 43 per cent of (8,000 gm. (body weight)-340 gm. (amputated leg)).

Increase in total carbohydrates in muscle ( $3,300 \times 0.56$  per cent) 18.5 gm.

Total excess dextrose in blood..... 11.5 "

Total dextrose accounted for by blood and muscle..... 30.0 "

or 94 per cent of the amount injected.

Therefore, 6 per cent not accounted for.

From Table IV, which summarizes the experiments upon whole dead animals, it is seen that the blood sugar content never reached 4.4 to 4.75 per cent, which we estimate would have been the percentage if all the sugar injected had remained in the blood. The highest blood sugar percentage found was 3.85 per cent in the portal blood in Experiment LD 49; the lowest was 1.48 per cent (except the 1.30 per cent of the same experiment, which is not absolutely reliable because of the method used in obtaining this blood sample). The irregularity in the figures of Experiment LD 49 is due to the fact that the various samples were taken from different parts of the circulation. Although one cannot compute the total amount of sugar in the blood in the dead animal experiments with accuracy, it seemed desirable to obtain a basis of comparison with the other series. We therefore give the figures in the column, "Dextrose not accounted for by blood," estimating the blood at 7 per cent of the body weight and using the last blood sugar figure obtained (except in Experiment LD 49 in which the average was used). It is thus seen that from 30 to 71 per cent of the sugar injected was not present in the blood.

In four of these experiments muscle taken before and after the injections was analyzed, and the increase in total carbohydrates indicated that a considerable proportion of injected sugar was to be found in that tissue. In Experiment LD 49 the dextrose content of the muscle rose from 0.54 to 0.88 per cent in 83 minutes, and the higher carbohydrates from 0.15 to 0.83 per cent. This increase in total carbohydrates of the muscle<sup>28</sup> accounted for practically the entire 30 per cent of the injected sugar which was still unaccounted for by the blood. A similar result occurred in Experiment LD 54,

<sup>28</sup> The proportion of muscle in the body was assumed to be 43 per cent of the body weight.

as all but 6 per cent of the injected sugar was accounted for by the blood and muscle analyses together. Here the rise in muscle dextrose was from 0.38 to 0.64 per cent, and in muscle polysaccharides from 0.30 to 0.60 per cent, the time being about 2 hours. In fact this result was the most striking of all, for here the muscle contained about 58 per cent of the injected dextrose. In Experiment LD 51 the amount of sugar still unaccounted for after 42 minutes was reduced to 27 per cent by the muscle carbohydrates, the dextrose increasing from 0.29 to 0.60 per cent, while the higher sugars increased but little; namely, from 0.14 to 0.21 per cent. This was the shortest of the four experiments. In Experiment LD 53 there was very little increase in the dextrose of the muscle, from 0.33 to 0.45 per cent, and none in the polysaccharide content. As this was the longest experiment (2½ hours), bacterial activity might be thought of as an explanation of the finding of the smallest increase of carbohydrate in the muscle. The increase in the polysaccharide content of the muscles in three of the four experiments is possibly significant, indicating perhaps a condensation of dextrose, after death, within the muscles.

*Experiments with Intravenous Injections of Dextrose into the Anterior Parts of Dead Animals.*

In Table V are given the results of four experiments upon dead anterior animals. These experiments were similar to the preceding series except that, before causing the heart to stop beating, the aorta and vena cava were ligated near the diaphragm.

In these experiments we again find high blood sugar values, and again we may compare them with the values which we estimated would exist if all the injected sugar had remained within the blood vessels, making due allowance for fluid injected and for blood samples drawn. The calculations give from 7.3 to 7.9 per cent blood sugar. It is seen that the percentages of sugar are actually much lower; namely, 2.24 to 3.42 per cent, showing that a good deal of the sugar injected into these dead stumps left the circulation rapidly. From Experiments 50 and 58 it would seem that this occurred most quickly in the first 30 to 45 minutes and then proceeded more slowly.

In three of the experiments the muscles were analyzed before and after the injection, and it was found that the muscles were richer in

TABLE V.  
*Intravenous Injection of Dextrose into the Anterior Parts of Dead Animals.*

No. of experiment.	Interval between injection.	Dextrose injected (1 gm. per kilo).		After injection.				Muscle before injection.		Muscle after injection.		Dextrose not accounted for. Per cent of total amount injected.	
				Time.	Blood sugar.	Time.	Blood sugar.	Dextrose.	Polysaccharides.	Time.	Dextrose.	Polysaccharides.	
	min.	gm.	cc.	min.	per cent	min.	per cent	per cent	per cent	min.	per cent	per cent	per cent
LD47*	2	27.0	135	15	3.27	43	3.07	0.47	0.57	128	1.03	0.57	55
LD50*	42	23.0	115	18	3.42	68	2.92†	0.44	0.39	88	0.74	0.42	58
LD58.	0	31.7	156	21	3.39	70	2.24	0.52	0.43	81	0.84	0.51	73
LD59.	2	36.0	180	30	2.32	60	2.34						71
													52

\* Thoracic duct ligated also.

† This sample of blood was obtained from the aorta after tying the inferior and superior venæ cavæ.

dextrose after the injection than before. The percentage of muscle dextrose rose from 0.47 to 1.03, from 0.44 to 0.74, and from 0.52 to 0.84 per cent, respectively. The figures given in the last two columns of the table were obtained on the assumption that the injected sugar reached only half the body, and further that this half contained the same proportions of blood and muscle as the whole body; namely, 7 and 43 per cent, respectively. The first of these two columns shows that a large proportion of the injected sugar had disappeared from the circulation. In Experiment LD 47 about 55 per cent was estimated to have left the blood in 15 minutes. In the other three experiments, each lasting about 70 minutes, the amount of sugar lost from the blood was about 58, 71, and 73 per cent. From the last column of the table we see that even when we deduct the sugar found in the muscle tissue there is still from 30 to 56 per cent not accounted for. In none of these experiments were we able to account for practically all the injected sugar by the muscle and blood analyses, as in two of the experiments on the whole dead animals.

The increase in the higher carbohydrates of the muscle in these experiments is negligible. In Experiment LD 50 there was no increase; in Experiment LD 58 the increase was from 0.39 to 0.42 per cent; and in Experiment LD 59 from 0.43 to 0.51 per cent. These results are in contrast with those found on the entire dead animals (Table IV) in which there was a considerable increase in the muscle polysaccharides in two experiments and a small increase in another. This difference may perhaps be brought into connection with the observation made by Levene and Meyer;<sup>36</sup> namely, that the pulp or juices of various tissues of the dog are able to convert dextrose into a higher carbohydrate if they are activated by spleen juice. In the anterior animal such a mixture of the products of the spleen and tissues by means of an artificial circulation is excluded.

#### DISCUSSION.

Large amounts of dextrose were injected intravenously into normal dogs. A variable proportion (an average of about 60 per cent) was eliminated by the kidney during the injection and the 90 minutes

<sup>36</sup> Levene, P. A., and Meyer, G. M., *Jour. Biol. Chem.*, 1912, xi, 353.

which followed. By the end of this time the percentage of sugar in the blood had fallen to, or nearly to, the level found before the injection. Consequently about 40 per cent of the injected sugar was not accounted for by the blood and urine analyses. When the same amount of sugar was injected into nephrectomized dogs, the blood sugar, while rising somewhat higher in the course of the experiment, fell to its original level as quickly as in the normal animals. Therefore nearly all the sugar had left the blood in the same length of time without the aid of the kidneys. When dextrose was injected intravenously into animals which had practically no circulation posterior to the diaphragm, the blood sugar did not reach the height which it would have attained if all the sugar had remained in the blood vessels. Since the dosage, however, was based on the weight of the entire animal, and as these dogs had only about half their circulation, the dosage was really about twice as great as in the first two series. For this reason an exact comparison cannot be made. Nevertheless, it was seen that the blood sugar gradually fell and that from 74 to 91 per cent of the injected sugar had left the circulation during the injection and the after-period, which was usually not more than 1 hour. Even in dead animals the blood sugar did not rise to nearly the percentage it would have reached if the injected sugar had remained in the blood vessels. Here, too, the percentage gradually fell, showing that from 30 to 71 per cent had left the blood without any vital forces taking part. In the last series, the dead anterior animals, a similar result occurred; from 55 to 73 per cent of the injected sugar could not be accounted for by the blood.

All these experiments show that the injected sugar disappeared quickly from the circulation. The first question that arises is: Is all this sugar rapidly burned up by the organism? The oxidation of carbohydrate in the animal body is usually tested by determining the respiratory quotient, a rapid rise of which to or near to unity being taken as an indication of sugar combustion. This has not been done by us, but it is well known that after the ingestion or injection of dextrose into the normal animal a rise in the respiratory quotient occurs. There is, however, no reason to assume that a nephrectomized animal will burn dextrose twice as fast as a normal animal. Yet this would have to be the assumption if we were to explain the

results of our normal and nephrectomized series on the basis of a rapid combustion. Tangl,<sup>27</sup> indeed, has found that after nephrectomy the gas exchange is diminished for  $3\frac{1}{2}$  to 4 hours, after which it begins to rise slowly. It therefore seems that a nephrectomized animal does not have a greater metabolism than a normal one during our experiments and probably does not oxidize more sugar. Another argument against rapid combustion is the disappearance of large amounts of sugar from the blood of dead animals. Here it can hardly be a question of increased metabolism.

The transformation of dextrose into glycogen in the liver is undoubtedly only a small factor in the disposal of intravenously injected glucose. This is evident from the work of Harley<sup>18</sup> and, more especially, of Freund and Popper.<sup>24</sup> The latter analyzed lobes of the liver before and after dextrose injection, and, when using a dosage like ours, found no increase in the glycogen content. Our experiments upon anterior animals, in which dextrose disappeared from the blood stream when all the abdominal viscera were cut off from the circulation, demonstrate clearly that the glycogenic function of the liver is not of great significance in tracing the fate of intravenously injected dextrose.

Undoubtedly one of the most important factors is the passage of the sugar into the surrounding soft tissues. In two experiments upon the dead entire animal practically all the dextrose not found in the blood was accounted for by the increased amount of sugar in the muscles. In the other experiments upon dead whole animals and dead anterior animals, from 9 to 38 per cent was estimated to be present in the muscles, and in three living anterior animals the estimations for muscle sugar were 5, 9, and 32 per cent of the amount injected. Evidently, a large and variable proportion of the injected dextrose simply passes through the capillaries into the muscle tissue. No doubt, the same process occurs in other parts of the body, more especially in all the soft tissues. The rapid disappearance of sugar from the blood is therefore, to a large extent at least, not difficult to explain.

However, the passage of dextrose into the tissues is not the only cause for its disappearance, for even in the dead animal experiments

<sup>27</sup> Tangl, F., *Biochem. Ztschr.*, 1911, xxxiv, 1.

we could not account for all the injected sugar in any of the anterior experiments or in two of the four experiments upon the dead whole animal. It is possible that some part of the dextrose is converted into higher carbohydrates in certain tissues. In fact some evidence, presented in the foregoing pages, indicates that this does occur in the muscles. In the living anterior experiments an increase in the higher carbohydrates of muscle occurred in two out of the three cases in which the muscle was analyzed. The same result was obtained in the dead whole animals in three out of four tests, while in the dead anterior animals there was little or no formation of polysaccharides. Apparently a condensation does occur in the muscles. But this does not account for any more of the sugar which has disappeared from the blood because it has already been included in our estimations. It may be that such a reaction occurs to the same or to a greater extent in other tissues and, in that way, a large part of the missing fraction would be accounted for.

The fact that in the dead whole animal a considerable condensation to a higher carbohydrate occurred in the muscles, while in the anterior ones very little was evident, suggests that this reaction may be influenced by an intra-abdominal organ. In the living anterior animals, however, a similar polymerization was found. To harmonize this with the above suggestion one would be obliged to assume that the almost insignificant amount of circulation still existing posterior to the diaphragm in living anterior animals suffices to bring from the abdomen the substance required to aid in the condensation. In the dead anterior animals the artificial circulation maintained at times by massaging the heart would not be enough to bring this about.

As other investigators have suggested, it is possible that a part of the injected dextrose is broken down to lower incompletely oxidized compounds. It would therefore not be entirely burned and at the same time, it would not be found as dextrose. We have no evidence to offer on this point.

#### SUMMARY.

1. As has been found by other investigators, when a large amount of dextrose is injected intravenously into a normal dog it disappears from the circulating blood in about 90 minutes after the end of the



injection. Varying amounts (an average of 60 per cent) are excreted in the urine.

2. Even in nephrectomized animals the same quantity will leave the circulation in the same length of time as in normal animals.

3. This phenomenon seems to be, at least to a great extent, independent of vital processes, since dextrose, after intravenous injection into dead animals, is found to leave the blood rapidly.

4. The phenomenon is independent of the important abdominal organs, for it also occurs in animals (living or dead) in which the aorta and inferior vena cava have been ligated near the diaphragm, thus abolishing most of the circulation posterior to the diaphragm.

5. The fact that a considerable amount of the sugar passes from the circulation into the surrounding tissues was established by finding an increase in the carbohydrates of the muscle tissue. This was done in the case of the living anterior animals and in the whole and anterior dead animals. In most of these experiments there was also evidence of the formation of polysaccharides in the muscle tissue.



# THE FUNCTION OF THE KIDNEY WHEN DEPRIVED OF ITS NERVES.\*

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PLATES 81 TO 83.

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The question as to what may be the function of a kidney excluded from all nervous influences is not settled at present, in spite of many experimental attempts by various observers. The reason for this lies in the fact that none of the methods employed to solve the question have been free from criticism. That the kidney is plentifully supplied with nerves is well known; but that these have any further function than that of vasomotor control has not been proved. Attempts to demonstrate secretory fibers to the kidney in either the vagus or splanchnic nerves or their branches have given only conflicting evidence.

Removal of all the nerve filaments at the renal hilus by dissection would exclude the kidney from the nervous system, so that its function could be compared with that of a normal organ. But on examining the situation more closely one finds that such method of nerve removal has been found most uncertain in its results.<sup>1</sup> This is due to the fact that the nerve filaments supplying the organ not only lie in a network closely applied to the renal vessels, but also run partly within the walls of these vessels. It has therefore been suggested to use some chemical means of destruction, such as painting with carbolic acid after the dissection. But even under these circumstances one is never absolutely sure that all nerves have been removed. Also, in such thin walled vessels as the renal vein, car-

\* Presented in abstract at the meeting of the Federation of American Societies for Experimental Biology, Boston, Dec. 26, 1915.

<sup>1</sup> Cohnheim, J., and Roy, C. S., Untersuchungen über die Circulation in den Nieren, *Virchows Arch. f. Path. Anat.*, 1883, xcii, 443.

bolic acid extensively used may cause a local reaction leading to thrombosis. For these or other reasons, none of the evidence thus far brought forward in regard to the function of such kidneys is convincing.

It has been shown by workers on the methods and possibilities of blood vessel suture that a kidney removed from the body of an experimental animal, and later reimplanted by restoration of the circulation, is able to support life in a presumably normal fashion. With one exception, however, all such investigators have been engaged in demonstrating the possibility of the operation, and have not concerned themselves with the detailed physiological function of the organ.

In the present investigation it was desired to examine in detail the function of a kidney which had been removed from the body and subsequently replaced. By this method it is certain that the organ is entirely outside the sphere of all nervous influences for a time at least, if not permanently. Furthermore, the response of such a kidney to the various functional tests gives at least indirect evidence on the question of secretory innervation.

#### HISTORICAL.

Asher<sup>2</sup> is one of the most recent investigators to attempt the demonstration of secretory nerves to the kidney. With his coworkers, Pearce and Jost, he has made observations which seem to show that the vagus nerve carries secretory fibers, while the splanchnic carries inhibitory ones; at least so far as the water output of the kidney is concerned. Jungmann and Meyer,<sup>3</sup> after cutting the splanchnic, found an increase of urine and of sodium chloride delivered from the kidney on the same side as the severed nerve. Rhode and Ellinger,<sup>4</sup> however, obtained different results, which led them to believe that the splanchnic has an inhibitory action. The difficulty seems to lie in the fact that in the methods

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<sup>2</sup> Asher, L., Die Innervation der Niere, *Deutsch. med. Wchnschr.*, 1915, xli, 1000. Asher, L., and Pearce, R. G., Die sekretorische Innervation der Niere, *Ztschr. f. Biol.*, 1914, lxiii, 83. Jost, W., Die sympathische Innervation der Niere, *Ztschr. f. Biol.*, 1914, lxiv, 441.

<sup>3</sup> Jungmann, P., and Meyer, E., Experimentelle Untersuchungen über die Abhängigkeit der Nierenfunktion vom Nervensystem, *Arch. f. exper. Path. u. Pharm.*, 1913, lxxiii, 49.

<sup>4</sup> Rhode, E., and Ellinger, P., Über die Funktion der Nierennerven, *Zentralbl. f. Physiol.*, 1913-14, xxvii, 12.

employed to elucidate the problem, the circulatory effects of vagus or splanchnic stimulation completely mask other possible effects.

More recently Pearce<sup>6</sup> has employed the method of Barcroft in which the oxygen consumption of the kidney is used as the index of cellular activity, and he has been unable to confirm his previous work done with Asher.

Transplantation or reimplantation of the kidney has been found surgically possible by Carrel, Stich, and a few others.<sup>6</sup> The most recent worker on this subject has been Lobenhoffer. His is the only work which attempts in any detailed way, to study the function of a kidney so treated. He united the severed renal vessels to those of the spleen in dogs, and was successful in ten instances. After removal of the other kidney, the water and salt output, as well as that of lactose and sugar caused by phloridzin, was studied. His results show that such a kidney is able to meet not only the ordinary demands of life, but also the excessive ones set up by the experimental injections. Further details of his work will be discussed later.

Zaaijer<sup>7</sup> has recently reported the survival and complete health of a dog bearing a single kidney, which had been transplanted to the iliac vessels 6 years previously.

### *Method.*

The experiments were carried out on large dogs of both sexes. After examination of the various regions where the kidney might be placed, it was decided not to transplant the organ, but to reunite it to its own severed vessels; for in this way the nearest approach to normal physiological conditions is secured.

Although the left kidney of the dog is somewhat more accessible than the right, and has slightly longer vessels, the renal artery on the left is often bifurcated, or even may leave the aorta as two separate vessels. This reduces the caliber of the arteries and doubles the amount of time spent in sewing. The right side was chosen, therefore, in nearly all instances.

<sup>6</sup> Pearce, R. G., and Carter, E. P., The Influence of the Vagus Nerve on the Gaseous Metabolism of the Kidney, *Am. Jour. Physiol.*, 1915, xxxviii, 350.

<sup>6</sup> Carrel, A., Doppelte Nephrektomie und Reimplantation einer Niere, *Arch. f. klin. Chir.*, 1908-09, lxxxviii, 379. Stich, R., Über Gefäß- und Organtransplantation mittelst Gefäßnaht, *Ergebn. d. Chir. u. Orthop.*, 1910, i, 1. Lobenhoffer, W., Funktionsprüfungen an transplantierten Nieren, *Mitt. a. d. Grenzgeb. d. Med. u. Chir.*, 1913, xxvi, 197.

<sup>7</sup> Zaaijer, J. H., Dauerresultat einer autoplastischen Nierentransplantation bei einem Hunde, *Beitr. z. klin. Chir.*, 1914, xciii, 223.

Ether was used by the intratracheal method in all the experiments. This method of administration is important, because by its use the respiratory movements can be reduced to a minimum, or even abolished. A motionless field is thus obtained which is of great aid in accurate and speedy sewing.

The animals were prepared for operation by shaving over the abdomen and far down onto the flanks. This area was then made sterile by soap and water, alcohol, ether, and tincture of iodine. A catheter was placed in the bladder, and in the case of male dogs, was carried off to the left side where it remained during the operation. The surgical asepsis was in every respect as strict as possible; gowns and gloves were worn, although during the actual suture of the blood vessels the gloves had to be removed, following which the hands were carefully coated with sterile vaselin.

*Transverse Abdominal Incision.*—Incision is made transversely across the abdomen just above the level of the umbilicus, and extending from the outer border of the left rectus abdominis across the right side down to the erector spinæ group of muscles (Fig. 1). This is well shown in the illustrations.\* Failure to prolong the incision far enough towards the posterior wall of the abdominal cavity prevents easy access to the whole kidney region, and makes the suture difficult or impossible.

The abdomen is entered in layers, careful attention being paid to hemostasis. After incision of the skin and subcutaneous tissues, the fascia of the external oblique is picked up on the right, at the outer border of the rectus. It is lifted up, incised, and the incision prolonged well toward the flank. The internal oblique is similarly treated. The rectus is dissected bluntly from its underlying sheath as far as the middle line, when it is cut through. The left rectus is similarly divided. After entering the abdomen through the linea alba, the transversalis can be quickly cut down in either direction (Fig. 2). The wound gapes widely and needs no retraction. All the intestines

\* This method of entrance to the abdominal cavity is of great aid in the performance of other operations, such as Eck fistula and those involving the biliary passages or adrenal glands. It is illustrated not because the procedure is in any way new, but because I have found that its value is not generally recognized by laboratory investigators.

except the duodenum and descending colon, are then lifted out of the abdomen to the left, where they lie covered by silk handkerchiefs impregnated with liquid vaselin (Fig. 3).

*Preparation of Kidney and Renal Vessels for Suture.*—After section of the peritoneum about the kidney, it is lifted out and turned toward the middle line of the body where, surrounded with gauze, it is gently held by the assistant. In this position the renal artery comes first to view, and it is carefully cleaned as far as the aorta of all surrounding tissue and nerves. This is done by blunt dissection and by wiping with dry gauze. The kidney is allowed to fall back into place, and the vein is similarly cleaned as far as the vena cava. The ureter is next stripped downward for about 6 or 7 cm. The field is then carefully washed with salt solution, every bleeding point caught and tied, and after drying, the whole is coated with liquid vaselin, including the kidney and its vessels. The two rubber-covered *serrefines* are then placed on the artery, two others on the vein, and the vessels are cut between them. The ureter is cut long. The kidney is then removed from the body and placed on a clean gauze pad. The gloves are now removed.

*Vessel Suture.*—The ends of the severed vessels are washed quickly with salt solution from a bulb pipette, till every trace of blood is removed. They are then coated with liquid vaselin. The adventitia is removed from the ends of the arteries in the usual way, after which the whole field within the abdomen is covered with vaselined silk handkerchiefs leaving only the stumps of the renal vessels protruding. These handkerchiefs are held in place by brass clips used by stationers, called "O.K. paper fasteners."

The most exacting and important part of the vascular suture lies in placing the three primary guide or tension stitches. This is made much easier by using a suture armed with two needles, one at either end. In this way the suture can always be passed from within the vessel outward. The circumference of the vessel ends must be accurately divided into thirds by these tension sutures.

The artery, being behind the vein, is sewed first, using an over-and-over stitch. This is followed by suture of the vein. The *serrefine* clamps are then removed from the vein and the vessel is allowed to fill under moderate pressure, rolling it a bit between the thumb

and finger. The artery is next freed while held between the thumb and finger so as partially to control the tension during the first few moments of blood flow.<sup>9</sup> After the vascular suture is ended, the ureter is reunited by the invagination method of Van Hook. Following this, the operative field is cleaned of blood, and the peritoneum readjusted by a few interrupted sutures about the periphery of the kidney. Special pains should be taken to see that the kidney is replaced high enough toward the liver so that there is no angulation of the vein.

*Closure of the Abdomen.*—The next step consists in closing the abdomen after the bowels have been replaced. This is made certain by using first a mattress suture of stout silk placed in the linea alba. After tying this, the severed abdominal muscles can easily be approximated in layers. A continuous suture is used for the transversalis, including at the same time the peritoneum. Mattress stitches are used for the recti, while the obliques are held by interrupted sutures. Closure of the subcutaneous tissue and skin completes the operation. Silk is used throughout the operation, both for ties and sutures.

The dog is placed in a metabolism cage so that the urine can be accurately collected and measured.

The operation is a difficult one, but after some experience it was found possible to restore the circulation in about an hour following its interruption. Forty-three dogs were subjected to operation, of which sixteen survived in suitable condition for further physiological observations.

#### *Examination of the Renal Function.*

The operated animals were divided into two series. In the first, the function of the reimplanted kidney was compared with that of the intact one, at periods varying from 2 days to 3 weeks after the primary operation. For this purpose the animal was anesthetized with paraldehyde, each ureter brought out onto the flank through

<sup>9</sup> The method of suture used is that elaborated by Carrel. Carrel, A., *La technique opératoire des anastomoses vasculaires et la transplantation des viscères*, *Lyon méd.*, 1902, xcvi, 859; *Anastomosis and Transplantation of Blood Vessels*, *Am. Med.*, 1905, x, 284; *The Surgery of Blood Vessels, Etc.*, *Bull. Johns Hopkins Hosp.*, 1907, xviii, 18.



lumbar incisions, and the urines were compared. This method has been described in a previous communication.<sup>10</sup>

The dogs of the second series were subjected to removal of the unoperated kidney at times varying from 5 to 14 days after the primary operation. The work of their remaining, reimplanted kidney was later examined and compared with that of control dogs, in whom a single nephrectomy had been done.

### *Series I.*

Two typical protocols will suffice to show the results of this series.

June 17, 1915. Dog 66. Weight 10,454 gm. Operation at 10 a.m.; reimplantation of right kidney.

June 18. Has made a good recovery. Blood urea is 0.302 mg. per liter.<sup>11</sup> Excretion of phenolsulphonephthalein given intravenously is 51 per cent in 2 hours.<sup>11</sup>

June 19. Given paraldehyde,<sup>11</sup> 1.7 cc. per kilo of body weight, by stomach tube, followed by 200 cc. of water. Ureters exposed in loins, and cannulae introduced delivering into test-tubes. Carotid blood pressure recorded on kymograph.

11.10 a.m. Urine appears without delay from each side. Collected for 1 hour.

12.10 p.m. 5.0 gm. of sodium chloride in 20 cc. of water given intravenously. Good diuresis from each side; urine collected for 1 hour.

1.10 p.m. Animal killed. The blood pressure during the experiment varied from 128 to 138 mm. of mercury, except just after the injection of the hypertonic salt solution. The sutured vessels were free from obstruction.

Microscopic examination of the reimplanted kidney showed the capsular spaces wide for the most part, and the glomerular tufts containing a considerable amount

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<sup>10</sup> Quinby, W. C., and Fitz, R., Observations on Renal Function in Acute Experimental Unilateral Nephritis, *Arch. Int. Med.*, 1915, xv, 303.

<sup>11</sup> As was shown in the communication noted above (Quinby and Fitz<sup>10</sup>) the two most dependable tests of renal function are the excretion of phenolsulphonephthalein and the estimation of the blood nitrogen or blood urea. The urease method of Marshall (*Jour. Biol. Chem.*, 1913, xv, 487) was used. By this the normal amount of blood urea of the dog is found to lie between 0.200 and 0.380 or 0.400 mg. per liter. The phenolsulphonephthalein excretion was estimated by the method of Rowntree and Geraghty (*Jour. Pharmacol. and Exper. Therap.*, 1909-10; i, 579). Paraldehyde was used, because in proper dosage it causes neither diuresis nor lowered blood pressure. It must be used fresh, however, because it loses strength on exposure to the air, thus making the dosage uncertain.

of blood. The cells and nuclei of the tubules were well preserved except in a very few areas where a small amount of cellular desquamation had taken place. The blood vessels were everywhere dilated, but no evidence of thrombosis or infarction could be found. A considerable number of hyaline bodies were seen in the collecting tubules, evidently casts. The uninvolved kidney was normal in all respects. Its capsules showed active diuresis, but the blood vessels were not so evidently dilated as were those of the operated organ.

		Amount of urine.	Percentage of NaCl.	Amount of NaCl.*
		cc.		gm.
1st hr.				
Before diuresis	Right.....	5.0	0.90	0.045
	Left.....	2.5	0.55	0.013
2nd hr.				
After diuresis	Right.....	60.0	0.95	0.570
	Left.....	22.0	0.95	0.209

\* The chlorides were determined by the Volhard method at first; later by that of McLean and Van Slyke (*Jour. Biol. Chem.*, 1915, xxi, 361).

We see that here the kidney without nerves shows an increased function over the normal one, both before and after diuresis.

June 30, 1915. Dog 67. Weight 11,363 gm. Left kidney reimplanted.

July 12. Has made a good recovery. Urine free from albumin and sugar. Average daily amount, 195 cc. Given paraldehyde, 1.7 cc. per kilo of body weight, followed by 200 cc. of water. Ureters exposed on flanks and cannulated. Blood pressure tracing from carotid.

11.05 a.m. Flow from right kidney begins a little later than from left which started immediately on delivering ureter. Urine collected for 1 hour after flow from each was established.

12.16 p.m. 5.0 gm. of sodium chloride in 20 cc. of water injected intravenously. Marked diuresis.

1.20 p.m. Animal killed. Blood pressure average was 133 mm. of mercury. Renal vessels without trace of clot, though there were a few adhesions about the kidney. A sample of blood drawn just before death showed 0.354 mg. of urea per liter.

Microscopic examination showed that both kidneys were normal in all respects except that in the one previously reimplanted a few mitotic figures could be found in the tubular cells after careful search. These were evidently the sequel of previous cellular degeneration.

		Amount of urine.	Percentage of NaCl.	Amount of NaCl.
		cc.		gm.
1st hr.				
Before diuresis	Right.....	2.5	1.12	0.028
	Left.....	3.0	0.93	0.028
2nd hr.				
After diuresis	Right.....	40.0	0.82	0.328
	Left.....	36.0	0.96	0.345

In this experiment the operated kidney has a function about the same as that of the normal side.

These two protocols fairly represent the findings in this first series which comprised eleven dogs. For a period following operation varying from 10 to 14 days the denervated kidney shows an increased absolute function both for fluid and salt, as compared with the normal kidney. At times this increase is relative as well. This is true of the unstimulated organ, and especially so of the one subjected to the diuretic action of sodium chloride. Beyond this period, however, the balance is regained, so that each organ, operated and intact, divides the labor in very nearly equal parts.

We see here also that absence of the renal nerves abolishes the temporary inhibition of flow so often seen normally after the handling of the ureters necessary for their exposure in the loin. Urine from the denervated side always flowed immediately on section of the ureter; but in some instances the normal side showed an inhibition lasting for as long as 5 minutes. This is analogous to the temporary inhibition occasionally seen on passage of a ureteral catheter in man.

### *Series II.*

The following is a typical protocol.

Nov. 20, 1915. Large male, of Newfoundland type. Weight 15,900 gm. Intratracheal ether and reimplantation of right kidney. Circulation restored after being interrupted for 1 hour.

Nov. 23, 1915. Has made a good recovery; eats well and does not vomit.

Nov. 30, 1915. Has entirely recovered. Wound healed by first intention.

Dec. 4, 1915. Ether. Lateral incision in left flank through which the kidney was removed after ligation of its vessels with silk. Wound closed in layers.

Dec. 7, 1915. Has made an excellent recovery. Passes 320 to 340 cc. of urine daily. Blood urea, 0.456 mg. per liter. Phenolsuphonephthalein, 60 per cent in

2 hours. Urinary sediment shows a rare blood corpuscle; no casts; a few leukocytes. Sugar and albumin absent.

Dec. 11, 1915. Animal well. The 24 hour amount of urine has fallen somewhat and its concentration has increased.

Dec. 14, 1915. Has a rather marked balanitis, and does not urinate until the demand is imperative. After about 48 hours during which no urine was passed, voided a little over 700 cc.

Dec. 18, 1915. Balanitis has responded to irrigation with boric acid and animal is now well. Output of phenolsulphonephthalein, 50 per cent in 1 hour. No albumin or sugar.

Dec. 22, 1915. Intravenous injection of 500 cc. of normal (0.8 per cent) salt solution. Urine withdrawn by catheter at 30 minute intervals showed the following:

	Amount.	NaCl per liter.
	cc.	gm.
1st half hr.....	83	10.6
2nd " ".....	62	12.3
3rd " ".....	48	13.0
4th " ".....	11	17.2

The urine before diuresis contained 19.10 gm. of sodium chloride per liter. It is thus seen that the kidney responds quickly to diuresis and regains its equilibrium within a normal time limit.

Jan. 31, 1916. Given 2 gm. of lactose intravenously. At the end of 5 hours 1.72 gm. were found in the urine.

Feb. 7, 1916. The 24 hour amount of urine has been measured for 65 days, giving an average of 180 cc. It contains neither albumin nor casts.

Feb. 9, 1916. Blood urea 0.320 mg. per liter. 61 per cent of phenolsulphonephthalein is excreted in 1 hour. Animal seems to be perfectly normal.

Four other animals of this series were killed after having shown normal kidney function for a month or longer. In each instance the kidney showed microscopic evidences of some hypertrophy of the elements, which usually occurs after unilateral nephrectomy. In two instances the kidney also showed a small depressed scar in the cortex with sclerosis of the normal elements and infiltration by connective tissue. These areas seemed to be the result of small focal necroses caused by interruption of the blood supply during operation. They were never of any considerable size, so that the function of the organ remained uninfluenced.

The results of this second series show that the life of dogs having a single reimplanted kidney is maintained in a normal manner, as estimated by renal functional tests as well as by other more general methods of observation.

#### DISCUSSION.

The experiments of the first series show that the immediate effect of loss of nerve control over the kidney is a period of overaction. This occurs in all cases, and in the presence of apparent health, as judged by the general condition of the animal, by the normal content of the blood in urea, and by a normal output of phenolsulphonephthalein. This period exists for a varying time, but balance has always been restored by the end of 2 weeks. The kidney recently deprived of its nerves is without vasomotor control; the organ is tense and appreciably enlarged; its vessels are dilated, and following the increase of blood flow there is an increase of function over that of the normal organ. Resumption of tone on the part of the blood vessels brings again normal function.

The results here would seem to be analogous to those vasomotor changes occurring in the splanchnic area after section of the cord. Following this operation there occurs a marked dilatation of the mesenteric vessels, but in a short time vasomotor control is again established. Vascular tone may be resumed through the intervention of other more peripheral nerve ganglia, or the smooth muscle fibers of the vessel wall may possibly regain their tone without such intervention. Also, in the kidney there are ganglion cells, especially in the region of the renal sinus, which may be responsible for the resumption of vasomotor control. Although we know that fibers of the sympathetic type are able to regenerate much more quickly than are those of the peripheral nerves, that they should be able to grow to the renal blood vessels and resume control over them within 2 weeks after section seems improbable. Certainly nerve control by the normal pathways could never have been regained in Zaaier's dog whose kidney was sutured to the iliac vessels, or in those of Lobenhoffer who used the splenic vessels.

The time variation in regaining normal function is probably to be explained by the greater or less degree of surgical insult in the indi-

vidual case. No kidney showed normal function after being excluded from the circulation for longer than 1 hour and 20 minutes.

The second series of observations indicates that a single kidney which has been removed from the body and subsequently reimplanted, can maintain normal life for apparently indefinite periods. Also such a kidney is able to respond to the excessive demands made on it by the injection of various test substances. My results in this group of experiments fairly coincide with those of Lobenhoffer, except in a few details. He found that the 24 hour amount of urine passed by his dogs varied between 1,500 and 2,000 cc. This is quite unusual. Normal cage dogs in a large number of observations made by us, are found to pass from 200 to 450 or 500 cc. of urine daily, having a specific gravity of about 1.030. Of course this is but a rough average, since all our animals had water continuously at hand, and must have taken varying amounts from day to day. Their diet was of meat. I feel, therefore, that the continuous excretion of such large amounts of urine tends to suggest the absence of complete return to normal conditions.

Again, in the three infusion experiments reported by Lobenhoffer, his animals put out amounts of water and salt which varied widely, though the quantities infused were the same. In the few infusions done by me the resulting outputs were all within 10 or 12 per cent of each other.

The observations on the relative values of the different methods used for testing renal function made by Quinby and Fitz<sup>10</sup> showed that the estimation of the blood nitrogen, or that part of it composing the blood urea, and the output of phenolsulphonephthalein, together form the best means of measuring renal function. Further experience with these tests in clinical work by many observers has confirmed this opinion. I have therefore been content to follow the dogs of this second series by means of these tests, rather than by phloridzin or lactose, as did Lobenhoffer.

The above results, though they throw no direct evidence on any possible secretory function of either the vagus or splanchnic nerves, seem to suggest that if this exists it must play a minor and infrequent part. Under all the conditions produced both by my experiments and by those of Lobenhoffer, the denervated kidney has been

seen to react in an entirely normal manner. One may ask, therefore, if secretory nerves to the kidney are assumed to exist, under what conditions they are manifest; for no lack of such action seems to be demonstrable. Added to the inability of the present observations to show any failure of kidney function which might be ascribed to lack of secretory nerve influence, is the recent work of Cow,<sup>12</sup> who finds in the duodenal mucosa some substance which has a definite diuretic effect on the kidney by means of a hormone action.

It is probable that vasomotor conditions in the kidney, added to the chemical and hormone action of substances contained in the circulating blood, will be found entirely adequate to explain all variations and types of normal renal function.

#### SUMMARY.

1. By means of vascular suture it is possible to remove the dog's kidney from the body and later to restore it to its former position.
2. Such a kidney is removed from the control of the nervous system, at least for a time.
3. Examination of the function of a kidney so treated shows an initial period of overaction, as compared with that of the normal kidney.
4. This is followed by balanced action.
5. The more recent tests of renal function show that a single, re-implanted kidney is able to maintain normal life indefinitely.
6. The results of these experiments, together with the evidence already at hand, suggest strongly that secretory nerves to the kidney do not exist.

#### EXPLANATION OF PLATES.

##### PLATE 81.

FIG. 1. The transverse abdominal incision extending from the outer border of the left rectus muscle well down into the right flank.

##### PLATE 82.

FIG. 2. The oblique muscles and both recti have been divided and the abdomen is being opened by incision of the transversalis in a direction parallel to its fibers.

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<sup>12</sup> Cow, D., *Jour. Physiol.*, 1914-15, xlix, 441.

PLATE 83.

FIG. 3. The intestines have been withdrawn for the most part from the abdomen, and lie to the left covered by silk handkerchiefs. The kidney, with its vessels and ureter, lies well exposed. Above the kidney is seen a portion of one of the lobes of the liver. At its inner side lie the inferior vena cava, and the pancreas enclosed by a portion of the duodenum.



*Received*  
*16*

FIG. 1.

(Quinby: Kidney Deprived of Nerves.)



FIG. 2.

(Quinby: Kidney Deprived of Nerves.)



FIG. 3.

(Quinby: Kidney Deprived of Nerves.)



# A METHOD FOR OBTAINING SUSPENSIONS OF LIVING CELLS FROM THE FIXED TISSUES, AND FOR THE PLATING OUT OF INDIVIDUAL CELLS.

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PLATES 84 TO 87.

(Received for publication, January 15, 1916.)

The only cells of the mammalian body which lend themselves as individuals to accurate experimentation *in vitro* while yet alive are the blood cells, the cells of exudates, and the spermatozoa. In saying this we do not overlook the usefulness of tissue cultivation or of experiments with living tissue fragments, of the transplantable tumors for instance. But both means of study involve, not individual cells, but complexes of different cells, which can be standardized only roughly, and which cannot be broken up into their component elements or protected from confusing factors, such, for example, as are introduced by death and autolysis of the central tissue portions. These difficulties have led us to work out a method whereby living tissue cells can be obtained as individuals in suspension, and, if desired, can be plated out in a culture medium (plasma) just as are bacteria. After growth the cells can be liberated again, and again plated successfully.

The method consists, in brief, in the growth of tissue in plasma, according to Carrel and Burrow's modification of Harrison's technique, and the liberation of the new cells by digestion of the clot with trypsin. We had noted that if the serum of a growing tissue culture is replaced with Locke's solution at room temperature the cells of the growing strands that extend out into the medium sometimes contract into spheres, which may be separate or, when growth has been dense, loosely attached, side by side. The general outline of the culture is maintained because the cells are held in place by the fibrin network; and if serum is added and incubation renewed they again put forth processes, and, joining each other, again form strands. The problem

has been to cause the cells to contract and then to liberate them from the fibrin network. This is readily done with trypsin in Locke's solution (Fig. 1); and the resulting suspension can be freed by filtration of all but individual cells.

#### *Method.*

We have used the trypsin powders of Merck, Grübler, and Kahlbaum. It is necessary to free them as far as possible from the ammonium sulphate which constitutes the greater part of their bulk. According to Kirchheim,<sup>1</sup> the trypsin of Merck does not contain ammonium sulphate; but we have found it present in as great amount as in the other preparations mentioned. It should be got rid of by Kirchheim's method. The trypsin powder is shaken briefly in absolute alcohol and allowed to stand while the heavy sulphate settles out. The supernatant flocculus is collected on a filter, rapidly washed with ether, dried in the air, and dissolved in Locke's solution (Locke's modification of Ringer's solution, but without sugar). The yield from 2 gm. of the unpurified trypsin is dissolved in 98 cc. of Locke's solution. The cloudy, yellowish fluid is filtered, first through paper, then through a Berkefeld cylinder (N) to sterilize it, and is distributed in test-tubes and kept in the ice box. It loses very slowly its ability to digest and can still be used after 2 months. 3 per cent trypsin digests plasma clots more rapidly and does not harm most cells; but 5 per cent kills cells. Unpurified trypsin powders can be employed but the results are not so good.

The tissue from which cells are to be obtained should be cultivated preferably in plasma diluted with Locke's solution in order that the fibrin network to be digested shall be slight. A mixture of one part of plasma with three of Locke's solution is a medium suitable for most tissues. If there is need for a thick suspension of cells many bits of tissue should be grown. It is convenient to flood them in small Petri dishes with a thin layer of the dilute plasma. After clotting has taken place each dish is sealed to prevent evaporation, and placed in the incubator. A stout cord dipped in hot, sterile paraffin and thrust between the outer and inner rim of the dish, with one end

<sup>1</sup> Kirchheim, L., *Arch. f. exper. Path. u. Pharm.*, 1911, lxxi, 352.



left free, is useful for sealing. A pull on the free end will release the top of the dish.

When growth is established the trypsin solution, warmed to 37°C., is poured on, filling the dish above the plasma, and incubation is continued. In a few minutes some of the tissue fragments are free, and within about an hour the clot has disappeared and there remains a clear fluid containing numerous tissue particles. This is taken up with a pipette, stirred to break up any loose aggregations of cells, diluted with Locke's solution, filtered through sterile gauze, and centrifugalized. The fine, powdery, yellowish gray sediment will consist of discrete cells, nearly all of them alive. They can be washed repeatedly if need be. We prefer for this purpose the "gelatin-Locke's,"—Locke's solution containing  $\frac{1}{8}$  per cent of gelatin,—which, as Rous and Turner showed,<sup>2</sup> protects fragile cells against mechanical injury. If the cells are to be plated again in plasma they need not be washed, but after centrifugalization can be suspended in the Locke's solution used to dilute the plasma. Plating is done, as before, in Petri dishes.

### *Results.*

The cells liberated as individuals by trypsin are those which grow out into the medium in strands or a meshwork, or which wander out separately (connective tissue cells, endothelium (?), choroid, sarcoma, and splenic tissue cells). Thus far we have used successfully the tissue of rat and chick embryos, of rat and chicken tumors, and the normal tissue of young rats. Sheets of growing cells (epithelium) are not readily broken up. Whether individual epithelial cells can be liberated in this way is as yet uncertain. But small groups of epithelial cells are obtained, and bits of striated muscle which live for a brief period when plated again.

The individual cells become approximately spherical when in suspension and the nuclei also tend to, though less perfectly. The change in form is especially noteworthy in the case of elements which, when growing in culture, are stellate or of an attenuated spindle shape with an elongated nucleus. When freed, suspended in

<sup>2</sup> Rous, P., and Turner, J. R., *Jour. Exper. Med.*, 1916, xxiii, 219.

serum, and stained, such cells show no trace of the long protoplasmic processes which they had while growing. With Wright's stain certain of them derived from connective tissue and probably of fibroblastic and endothelial origin have a resemblance to the mononuclear series of the blood (Fig. 2). Their cytoplasm is basophilic. Other cells from the same source are three or four times the diameter of any blood element. These morphological features will be taken up in a later paper.

The freed cells, distributed in plasma as separate individuals and incubated, soon put forth processes and assume their original form. Bits of striped muscle from the embryo may round at the ends, thus gaining a leech shape, and put out short processes (Fig. 4). We have not observed them to proliferate. But the spindle-shaped and stellate cells of connective tissue, sarcoma, and the choroid coat of the eye multiply rather rapidly. If the cells are numerous the plate will show at the end of 24 hours a thick mesh- or feltwork consisting of elements once separate which have reached out and joined each other by means of attenuated processes (Fig. 3). The tendency of scattered cells thus to connect with each other again is striking. At the end of 48 hours the number of growing elements is greatly increased, not only by proliferation but by the "waking up" of cells previously spherical. If small masses of cells are present in the culture, as the result of incorrect filtration, growth from them may be almost explosive, each mass resolving itself into elements that radiate in every direction.

### *The Replating of Cultures.*

The limits of the method have not yet been reached. The freed and plated cells can be liberated anew after growth and successfully plated again in fresh plasma. To judge from our results, the process can be repeated indefinitely. Isolated cells of the chick's choroid continue to form pigment after they have been twice liberated with trypsin and twice replated (Fig. 5).

Cells that have been growing in tissue cultures for more than 24 hours when freed and examined in suspension show, as a rule, fat droplets, and corresponding vacuoles when fixed and stained in the

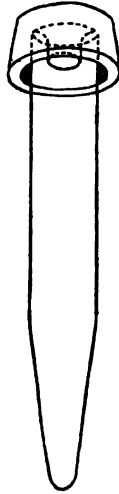
spherical state. Fat droplets have often been noted in tissue cultures and their source is to some extent known.<sup>3</sup> But they are much less prominent in the culture with its extended cells than in the freed, contracted elements. We wish to emphasize the fact that they develop very early, even when growth is taking place in a dilute plasma medium. Only during the first 24, or rarely the first 48 hours, do the cells appear absolutely normal. Later the culture consists for the most part of abnormal elements. This is true also of the freed and plated cells. It follows that replating should be carried out at least every 48 hours.

#### *Technical Difficulties.*

The initial cultures must be free from bacteria if the cells are to be replated after their liberation. For the tryptic digestion liberates not only tissue cells but bacterial colonies, and a single one of these latter can by its dispersion ruin all of the new plates. For this reason it is best to cut up the tissue to be grown, in a sterile, glass-sided box, closed with pieces of rubber dam at the ends, through apertures in which the instruments and tissue are introduced, and the hands thrust, encased in sterile, rubber gloves. A small, glass hood with cloth sides will do nearly as well, and it is useful for the replating of cultures. Needless to say a single contamination at any time will ruin a sequence of plates. If the cells are to be used in suspension it is of less importance.

The centrifugalization to bring down tissue cells brings down also fine débris such as bits of cotton, particles of dust, etc., from the fluid. By the time cultures have been twice digested and plated, enough of this will have been collected to mar their appearance, unless special care is taken. Such care consists in the use of well filtered fluids, and centrifuge tubes closed with corks instead of cotton or gauze stoppers. Much time can be saved if the corks are hollowed to fit over the end of the tube, but with a central core to prevent dislodgement (Text-fig. 1). They may be boiled or autoclaved. The central core should be rather short in order that it may remain uncontaminated when the cork is placed on an unsterile surface.

<sup>3</sup> Lambert, R. A., *Jour. Exper. Med.*, 1914, xix, 398.



TEXT-FIG. 1. Centrifuge tube closed with an easily removable cork designed to keep the contents sterile.

#### SUMMARY.

Individual, living, tissue cells can be obtained in suspension by digesting with trypsin the clot of growing tissue cultures. Under these circumstances the living cells assume a spherical form. When washed and plated in fresh plasma they put out processes and proliferate. After growth in the new plates has occurred the digestion and plating can be repeated. The limits of the method have not yet been reached. We are at work on a number of the problems which it has opened up.

#### EXPLANATION OF PLATES.

##### PLATE 84.

FIG. 1. Edge of a culture undergoing digestion with trypsin. The cells have begun to contract into spheres. (Chick embryo.)

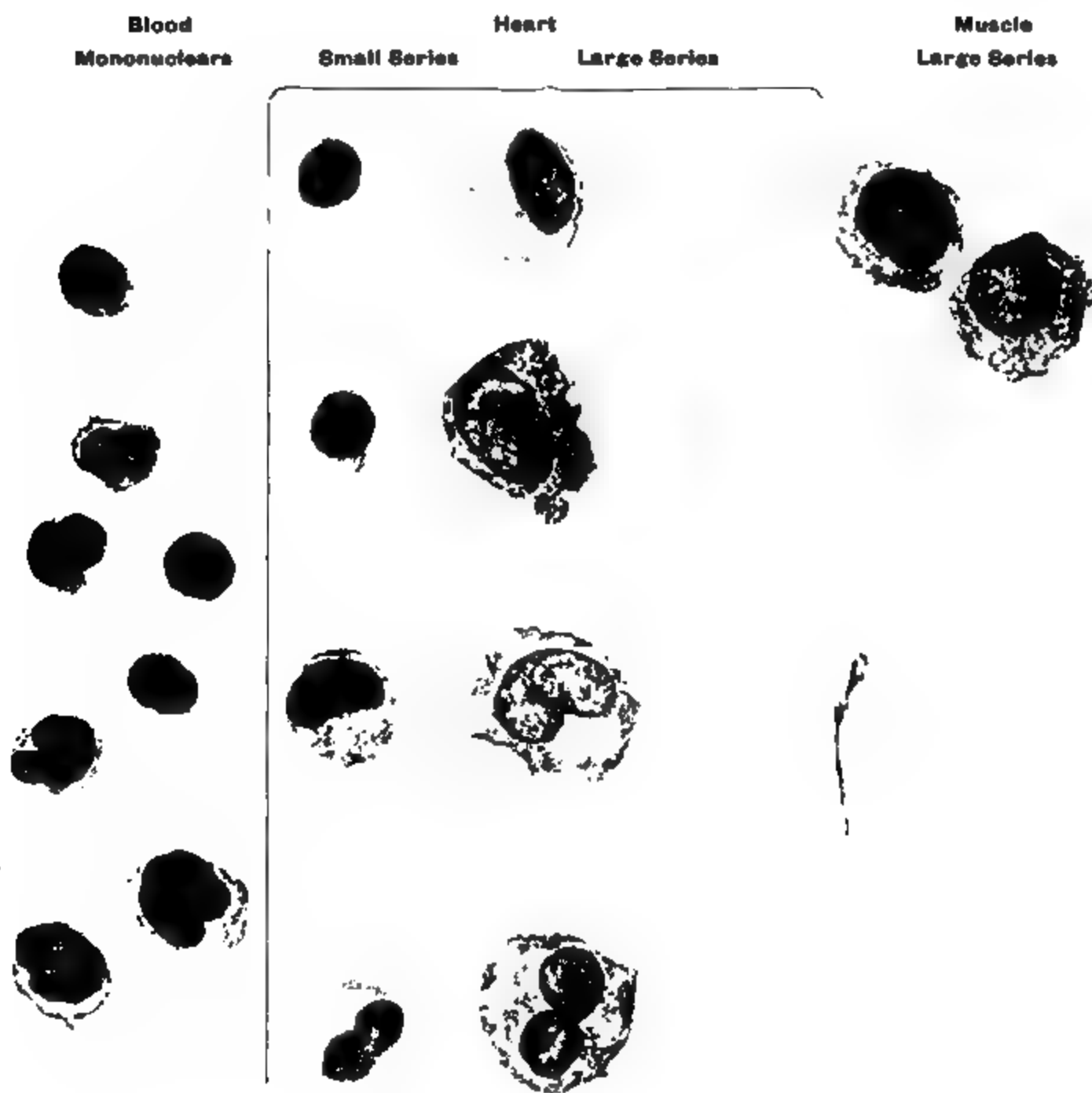
##### PLATE 85.

FIG. 2. Connective tissue and endothelial (?) cells liberated from cultures of the heart muscle and abdominal muscle of a 3 day old rat. Mononuclear cells from the blood of the same animal. Wright's stain. All the cells are drawn to the same magnification.

FIG. 1.

(Rous and Jones: Living Cells from Fixed Tissues.)





*Living cells from fixed tissues*







FIG. 3

(Rous and Jones: Living Cells from Fixed Thymus.)



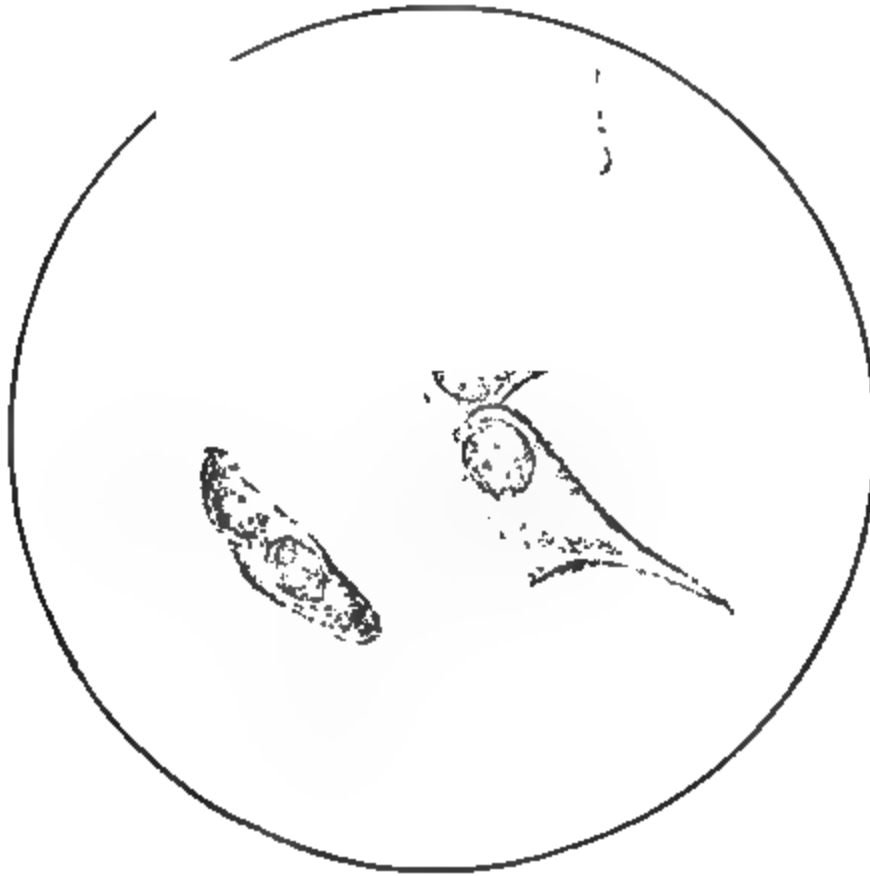


FIG 4

FIG. 5.

(Rous and Jones: Living Cells from Fixed Tissues.)



The cell marked *a* has ingested two red cells. One cell of the muscle series shows vacuoles resulting from a fatty change, and another has attached to it undigested fibrin threads.

## PLATE 86.

FIG. 3. Meshwork formed by the anastomosis of connective tissue cells liberated by trypsin and plated as separate individuals. (Chick embryo.)

## PLATE 87.

FIG. 4. Striped muscle from a culture incubated 24 hours after liberation by trypsin and replating.

One fragment of muscle, with sharp-cut ends has not grown and has undergone fatty change. But the others give evidence of life, as shown by their change in form, and one has put forth a process. (Rat embryo.)

FIG. 5. Cells from the chick's choroid growing after two liberations with trypsin and two replatings. The formation of pigment is going on actively.



## THE PURE CULTIVATION OF SPIROCHÆTA ICTERO-HÆMORRHAGIÆ (INADA).

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PLATES 88 AND 89.

(Received for publication, December 1, 1915.)

Numerous theories exist regarding the etiology of Weil's disease. In June 1914 Inada first observed the occurrence of a spirochæta in this disease, which he designated *Spirochæta icterohæmorrhagiæ*. The spirochæta has since been identified as the etiologic agent of Weil's disease. According to Inada<sup>1</sup> the spirochæta does not grow in solid or semisolid culture media except in a slightly modified medium originally employed by Noguchi for the cultivation of the spirochætæ of relapsing fevers, thus necessitating the use of a piece of fresh tissue as one of the ingredients.<sup>2</sup> The pure culture of this organism produces no odor and is invisible to the naked eye on account of the transparency of its colonies. The spirochætæ grown in these cultures are few in number (Table I), although Inada reports that occasionally there were as many as fifty individuals in a field. Judging from the comparatively poor growth of the spirochætæ, it may be assumed that the method employed by Inada is still far from perfect. Contrary to the experience of Inada, we were able to cultivate the organism in solid or semisolid media, and we have succeeded also in obtaining the culture in a fluid medium.

*Material for Cultivation.*—Guinea pigs are inoculated intraperitoneally with blood or urine from a person suffering from Weil's disease, and after the animals have developed unmistakable symptoms of the disease, *i.e.*, within 3 to 7 days after inoculation, a sufficient amount of blood is withdrawn, under general anesthesia, from the heart of the animals by means of a sterile syringe, the usual aseptic

<sup>1</sup> Inada, R., *Jour. Exper. Med.*, 1916, xxiii, 377.

<sup>2</sup> Noguchi, H., *Jour. Exper. Med.*, 1912, xvi, 199.

precautions being observed. The samples of blood thus obtained are examined under the dark-field microscope for the spirochætæ and those with a positive finding are selected for the purpose of cultivation. The blood may be used at once or after being kept in a sterile test-tube for several days at room temperature, during which time the spirochætæ increase in number in the majority of instances. The cultures are made in a fluid, a semisolid, or a solid medium.

A small piece or an emulsion of the liver or kidney of animals that have died of experimental Weil's disease may also be used for the purpose of cultivation, but we prefer blood from the heart on account of the comparative ease with which other bacteria can be prevented from entering the culture.

TABLE I.

Date.	No. of spirochætæ in one field.	Date.	No. of spirochætæ in one field.
<i>1915</i>		<i>1915</i>	
May 14.....	1- 2	June 15.....	14-15
" 19.....	1- 5	" 18.....	3- 4
" 24.....	1- 8	" 21.....	—
" 26.....	1-18		
June 2.....	1- 4		
" 4.....	4-10		
" 8.....	8-10		
" 12.....	20-30		

*Varieties of Culture Media and Mode of Cultivation.*

(1) *Semisolid and Solid Media.*—Blood agar and blood gelatin were found to be the most satisfactory media. Blood agar is prepared by mixing one part of the blood from a normal guinea pig or a man with one or two parts of the ordinary nutrient agar while the latter is still in a fluid state at a temperature of about 50°C. The blood gelatin is prepared by mixing one part with two to four parts of the blood at a temperature of about 25-30°C., at which the gelatin is still fluid. As soon as the media are prepared, and before they become solidified, the inoculation is made by adding a drop of the infected blood and distributing it well in the media by stirring the tubes. The inoculated tubes are now placed in a temperature any-



where between 15° and 37°C. As a rule, a layer of fluid paraffin about 2 cm. deep is poured into the culture tubes in order to cover the surface, although an abundant growth may be had without the addition of the paraffin oil. It may be remarked that these blood media appear to be a much deeper red as well as opaque near the bottom, as the erythrocytes gradually sediment before the agar or gelatin becomes solidified.

The culture tubes are best left undisturbed for at least 10 to 14 days. The greater the amount of infected blood introduced into the media, the more certain are the chances of obtaining a culture, for the reason that we thereby introduce more spirochætæ. According to our experience blood gelatin gives a better result than blood agar.

The commencement of growth in the solid media is not constant, but this usually takes about 1 week, after which the spirochætæ increase in number for the following 2 to 3 weeks, at which time the growth reaches its maximum. We therefore renew the culture by transplanting it into the new media every week or two. For this purpose the inoculation is made by the stab method.

The growth of the spirochætæ is accompanied by neither an offensive odor nor by the production of a gas and there is no liquefaction of the media.

*Morphology.*—The spirochætæ grown in our media show active movement under dark-field illumination (Leitz apparatus) and possess a number of small rectangular curves regularly set along the whole length of the organism. It is difficult to count the curves accurately while the spirochætæ are actively moving, but there are some with from two to three curves and some with as many as fifteen curves. The long specimens resemble *Spirochæta pallida*. The body of the spirochæta presents a granular or beaded appearance due to the unequal refractive power as manifested by different sections, the sections with a stronger refractive power being set alternately with those possessing a weaker power. The average organisms show more than fifteen granules and the size of the granules becomes somewhat smaller towards the extremities, which are drawn out into sharp points. In a resting specimen or before motion has begun one of the ends may assume a blunt appearance.

The length of the small specimens attains a quarter of the diameter of an erythrocyte, while that of the long ones is five times as long.

*Motility.*—The movements are sometimes forward, sometimes backward, and at times certain lateral motions are also noticed. The organism may shoot through the field with great rapidity or display a corkscrew or serpentine movement along the long axis. In a fresh preparation sealed with dammar and kept at room temperature, the spirochætæ become gradually sluggish and finally immobile within varying lengths of time, occasional mobile individuals still being discernible among them.

*Number of Spirochætæ.*—The number of organisms in a culture may vary according to the age of the latter, but they are generally innumerable, as they gather diffusely or in bundles, and it is impossible to count all the spirochætæ in a field (Fig. 1).

*Staining Reaction.*—The organisms do not take any of the ordinary aniline dyes, but assume a pinkish or pinkish purple color when stained with Giemsa's solution. The appearance of the stained spirochætæ differs considerably from the morphological features of living specimens (Fig. 2). They are much heavier near the middle and taper off into sharp points at both ends, thus resembling *Spirochæta refringens*. The spirochætæ in a stained preparation are much shorter than the organisms observed under the dark-field microscope. The organisms grow at any temperature between 15° and 37°C., but the optimum temperature is between 20° and 25°C.

*Mode of Development.*—In a solid culture where the inoculation of the spirochætæ is made in a fluid state the growth is diffuse throughout the media, but in a stab culture they multiply around the stab canal and then spread diffusely. In a young culture the spirochætæ are short and are found near erythrocytes or sometimes attached to them. As time passes the organisms grow longer and wander away from the red corpuscles to form masses or remain scattered. We are unable to decide whether the spirochætæ multiply by longitudinal or transverse division, but we have seen two specimens intertwined. In some instances two spirochætæ are seen to be united at one end, while a bundle of immobile specimens lying parallel may be seen to break up suddenly into units and to move away individually.

(2) *Fluid Media.*—Blood serum of man or ox diluted with an equal part of distilled water or undiluted ascitic fluid or pleural exudate is

sterilized by subjecting it to a temperature of 50–60°C. for half an hour for several successive days. Sterile tubes are each filled with 10 cc. of the fluid. Another way of preparing a fluid medium is to follow the method of Noguchi; namely, to add to the above fluid a small piece of kidney from a normal guinea pig and then to use the media after ascertaining the sterility by incubating them for 24 hours. Instead of the kidney, small amounts of coagulum of human or guinea pig blood may be used.

The inoculation of the fluid media is made by introducing one or two loopfuls of the infected blood containing the spirochætæ. The tubes are then placed in a temperature varying from 15–37°C. By this method we have succeeded in obtaining a good growth which was first noticed after 3 to 10 days by the appearance in the clear fluid of a light haze resembling a culture of *Spirochæta pallida*. Upon examination under the microscope numerous spirochætæ identical with those grown in a solid medium were found. Unfortunately, the culture died out in the second generation, probably owing to contamination with a bacillus of the *coli* type or to the lack of the red corpuscles in the fluid media. The transfer in this case was made on the 5th day and the spirochætæ died on the 5th day of the second generation.

A pleural exudate rich in fibrin seems to be the most suitable fluid medium for the purpose of cultivating this organism.

*Pathogenicity.*—For the purpose of determining the pathogenic property of the pure culture of the spirochæta we have inoculated a small quantity of the culture into the peritoneal cavity of a number of guinea pigs. In the course of 4 to 8 days after the inoculation the animals succumbed after the usual symptoms of the disease. The post-mortem showed all the characteristic lesions. From these animals we have recovered the same organism in pure culture. This experiment completes the links of evidence proving that the spirochæta in question is the causative agent of Weil's disease, and it shows that the pathogenicity of the organism is not noticeably diminished through artificial cultivation. The spirochætæ were found in sections of the liver of the guinea pig from which the culture was derived and of the guinea pig which showed the typical symptoms after receiving the inoculation of the culture (Figs. 3 and 4).

According to Ashizawa's experiment, blood serum from a patient once attacked by Weil's disease has a slight bactericidal action upon the spirochætæ cultivated by our methods.

#### CONCLUSIONS.

Pure cultures of the spirochætal causative agent of the disease known as Weil's disease, or febrile icterus, in Japan, have been obtained by us in a solid, a semisolid, and a fluid medium. The spirochætæ thus isolated remains pathogenic for guinea pigs for many generations. Up to the present time we have succeeded through the courtesy of Professor Nagayo, Dr. Konuma, and Dr. Ishihara, in cultivating three different strains.

The spirochætæ is a facultative anaerobe.

The solid and semisolid culture media possess one disadvantage, in that they are opaque on account of the addition of red blood corpuscles; but it is hoped that this drawback may soon be overcome by further studies. We shall report later the results of investigations regarding various questions in immunity as well as further details regarding the biological properties of the spirochætæ.

We wish to express our gratitude for the many valuable suggestions and the assistance which Professor Dohi and Dr. Noguchi rendered us during the execution of the present work.

#### EXPLANATION OF PLATES.

##### PLATE 88.

FIG. 1. Dark-field view of *Spirochæta icterohæmorrhagiæ* from a pure culture in a semisolid blood gelatin medium. The dark spheroid bodies with a refractive ring are erythrocytes. The white, wavy, beaded lines represent the spirochætæ. Semischematic.

FIG. 2. A film preparation of *Spirochæta icterohæmorrhagiæ* from a pure culture in a semisolid blood gelatin medium. Giemsa's stain. Semischematic.

##### PLATE 89.

FIG. 3. Distribution of *Spirochæta icterohæmorrhagiæ* in the liver of a guinea pig in which typical symptoms and lesions had been produced by injecting a pure culture of the organism. Levaditi silver impregnation method.  $\times 1,000$ .

FIG. 4. A film preparation of liver emulsion obtained from a guinea pig which died of experimental Weil's disease produced by a pure culture on the sixth day. Giemsa's stain.  $\times 1,000$ .

FIG. 1.

FIG. 2.





(Ito and Matsuzaki: Cultivation of *Spirochaeta scierohamorrhagiae*.)





*The Journal of Experimental Medicine* will be glad to hear from persons who have copies of Vol. XII, No. 4, Vol. XVII, No. 1, and Vol. XIX, No. 1, to dispose of. The *Journal* will pay for these numbers the regular listed prices.

field. Arsenic, mercury, quinine, and certain dyes are perhaps the only substances which up to now have been regarded with assurance as leads for chemotherapeutic development. And of these, with the exception of optochin and perhaps of salicylic acid, all have been indicated for protozoan infections only, and there is promise that by the proper development of these substances the problem of the control of most protozoan infections will be successfully solved.

When we turn, however, to the cure of infections of purely bacterial origin the problem which confronts us is found to be more difficult of attack. Here not only the difficulty of procuring a suitable experimental infection as a test object, but the absence of clinical observations which might be regarded in the nature of leads which could form the basis for a rational chemotherapeutic procedure, makes it imperative from the start to seek for such leads. The successful chemical development of quinine which led to the discovery of ethylhydrocuprein already indicates that resource may be had eventually to the above mentioned protozoan leads. And it is a fact that perhaps with the exception of salicylic acid this drug is the first substance to have been used successfully in an experimental bacterial infection. This one fact creates the hope that chemotherapy may find a wider application in the control of bacterial infections. It is conceivable, however, that ultimate success may depend upon the finding of leads other than those which have been successfully used against protozoan infections.

It would seem that a wealth of material should lie ready among the numerous classes of organic substances which have been found to exert powerful bactericidal effects *in vitro*. But the failure of many of these when tried in experimental infections has led to the realization that besides a bacteria-killing property the fulfillment of certain other requirements is essential for the achievement of an internal antiseptis. Some workers believe that no indication of the probable effect of a substance *in vivo* can be discerned from its action *in vitro*, and that the successful control of an infection by chemical agents can be attained only by indirect means. This might occur, on the one hand, as perhaps in the case of atoxyl, by the chemical transformation *in vivo* of the injected drug into some active form. On the other hand, the substance might act through the protective mechanism of

the host either by increasing phagocytosis or by stimulating the production of immunity principles. It is, of course, probable that in some instances these phenomena might play a part and that a possible scheme of chemotherapeutic attack could be developed along these lines.

Owing to the ease of handling bacteria *in vitro* and the simplicity of the bactericidal tests it would be unwise because of former failures to condemn the *in vitro* method, at least as a means of initial orientation. If it were accompanied by certain parallel studies the *in vitro* method should do more than afford only orientating data. As pointed out by Bechhold and Ehrlich<sup>1</sup> and others in the past, besides a mere bactericidal power other conditions must be fulfilled before a substance may be considered even a possibility as a therapeutic agent, provided of course a direct action by the drug itself is in question.

Aside from the obvious conditions of solubility and relative non-toxicity, the drug once in the circulation, whether by direct intravenous injection or by absorption, must be maintained therein for a sufficient length of time and in sufficient concentration in order to unfold its *in vitro* effect. In other words, its free access to the foci of infection should not be completely obstructed.

To accomplish this it must not enter too rapidly into chemical or physical combination with the constituents of the tissues, of which the blood is a fair representative. It must not be too speedily eliminated. And, finally, it must not be too rapidly altered in any way by metabolic processes which would nullify its bactericidal character. There may be still other and less definite factors which separate the *in vitro* from the desired *in vivo* result. If, however, the *in vitro* bactericidal tests could be complemented by a parallel study of those properties of substances which would decide whether they could satisfy the above requirements *in vivo*, our choice of substances for the *in vivo* experiments could be in great measure controlled. A system, though somewhat arbitrary, would be substituted in an undertaking which would otherwise be directed by a haphazard and entirely opportunistic policy.

These considerations have convinced us that the procedure in the search for leads in the chemotherapy of bacterial infections may be

<sup>1</sup> Bechhold, H., and Ehrlich, P., *Ztschr. f. physiol. Chem.*, 1906, xlvii, 173.

logically systematized as follows: Substances which either by their general structure or by the possession of characteristic atomic groups are representative of as many types of organic substances as possible should be systematically selected for bacteriological and biological testing. Such facts as the bactericidal power and partial specificity for certain types, compatibility with tissue constituents (serum), and resistance to profound and rapid metabolic alteration should be noted and considered in the final interpretation of what in the chemical constitution of the substances is responsible for the observed biological behavior.

With organic substances there will be considerable difficulty in satisfying the last requirement. In the case of arsenicals and mercurials it is immaterial whether metabolization should occur, for the therapeutic characteristics of such compounds are elements. Their value may partly depend upon such metabolization. It does not seem improbable, however, that bactericidal substances may be found which, even though to a less degree than the arsenicals and mercurials, may be sufficiently resistant to metabolic changes to enable them to produce a sterilizing effect before they are disposed of by the host. The large number of pharmacologically active preparations must all persist long enough after administration to produce their physiological actions.

From the representative substances which have been found to possess the required biological properties, two classes of leads might be obtained: first, those substances which, like quinine, owe their bactericidal action to the general structure of the molecule; and, second, those which, like phenol, are bactericidal principally because of the possession of a certain atomic group. Once in the possession of *bactericidogenic*,<sup>2</sup> tissue-compatible molecules or side-chains, the same systematic development so successfully employed in the development of organic arsenicals by the alteration or addition of groups to the molecule might be here repeated in order to augment the specific bactericidal action, to detoxify it, or in some other way

<sup>2</sup> The word *bactericidogenic*, of obvious derivation, is employed in this and the following articles as a convenient term to express the property of certain chemical groups, when introduced into an organic molecule, of imparting bactericidal properties to that molecule.

furnish it with biologically desirable properties. In this way substances could be obtained which would form a rational basis for chemotherapeutic investigations.

The problem of the chemotherapy of bacterial infections and a possible scheme for its systematic attack have been discussed above in some detail with the purpose of affording a basis for a better understanding of the material which will be presented in the following papers.

From its nature this material will touch on but one phase of the above scheme and no claim is made of its complete realization. We shall present the results obtained in a systematic attempt to alter chemically the molecule of hexamethylenetetramine with the object of obtaining a class of bactericidal substances which could be employed in experimental infections. The use of this drug was inspired by the interest felt by Dr. Flexner in the possible application of some of its derivatives in the treatment of experimental poliomyelitis, and the material which will here be presented is but a part of a larger undertaking executed with it.

We shall attempt to show how, by the selection of a certain molecular group, namely hexamethylenetetramine, it has been possible to demonstrate its general bactericidogenic character. By the combination of this substance in the form of quaternary salts, in the manner to be described later, with a great variety of other molecular groupings a new class of bactericidal substances has been prepared<sup>3</sup> in which the bactericidal nature was principally attributable to the hexamethylenetetramine nucleus. On the other hand, the degree of this action was determined by the nature of the molecular groups added to hexamethylenetetramine. These added groups were likewise responsible for the partial specificity of certain of the preparations for particular bacterial species. This partial specificity did not favor one species alone, but all the species tested were found to be separately and specifically susceptible to some particular type of hexamethylenetetramine derivative. We must therefore conclude that the bactericidogenic character of hexamethylenetetramine ex-

<sup>3</sup> For the chemistry of these substances and the references to those prepared by others see Jacobs, W. A., and Heidelberger, M., *Jour. Biol. Chem.*, 1915, **xx**, 659, 685; 1915, **xxi**, 103, 145, 403, 439, 455, 465.

hibited in its quaternary salts is not specific but general in character. The specificity, however, is furnished by the proper choice of the molecular grouping added.

It will also be shown that a few of the hexamethylenetetraminium compounds which were tested were either not at all or but slightly inhibited by serum. A few, on the other hand, were found to be greatly inhibited by serum. The fact, however, that any one of the hexamethylenetetraminium salts is compatible with serum is enough to demonstrate the serum compatibility of the bactericidogenic hexamethylenetetramine portion of the molecule itself. We have here, therefore, a bactericidogenic, serum-compatible group. The remainder of the molecule determines the serum incompatibility of those substances the action of which was found to be inhibited by serum.

In the same way the toxicity relationships were found to be determined by the groups contained in that portion of the molecule added to the hexamethylenetetramine nucleus.

We can regard the material here presented merely as a beginning, but we feel that such a treatment of the problem as here presented may ultimately result in an accumulation of data which will be of value in the systematic search for substances which may be used in the control of experimental bacterial infections. Before passing judgment, however, on the chances offered by the further development of the quaternary salts of hexamethylenetetramine, the behavior of these substances in the animal organism should be studied in order to determine whether the bactericidogenic group in itself is sufficiently resistant towards metabolic changes. Otherwise these compounds as a class would be bactericidally inert *in vivo*.

# THE BACTERICIDAL PROPERTIES OF THE QUATERNARY SALTS OF HEXAMETHYLENETETRAMINE.

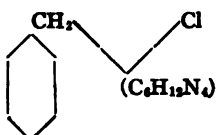
## II. THE RELATION BETWEEN CONSTITUTION AND BACTERICIDAL ACTION IN THE SUBSTITUTED BENZYLHEXAMETHYLENETETRAMINIUM SALTS.

By WALTER A. JACOBS, Ph.D., MICHAEL HEIDELBERGER, Ph.D., AND HAROLD L. AMOSS, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, March 1, 1916.)

Hexamethylenetetramine, as a tertiary nitrogen compound, reacts readily with benzyl chloride, bromide, or iodide, and their numerous nucleus substituted derivatives to form quaternary salts. The results obtained in the study of the bactericidal properties of such substances are the subject of the present communication. In these compounds, the structure of which may be represented as follows,



it is seen that by means of a  $\text{CH}_2$  side-chain the hexamethylenetetramine molecule is linked to a benzene nucleus. By the use of a great variety of substituted benzyl halides it was found possible to prepare for study a variety of hexamethylenetetraminium salts<sup>1</sup> in which the benzene nucleus was varied at will in the character, number, and position of the different atoms and groups introduced. By this procedure the opportunity was afforded of studying the effect of chemical constitution upon bactericidal action in a uniform series of substances.

Because the number of substances involved in the investigations

<sup>1</sup> For the chemistry of these substances and the references to those prepared by others see Jacobs, W. A., and Heidelberg, M., *Jour. Biol. Chem.*, 1915, **xx**, 659; 1915, **xxi**, 465.

described in these papers was so large, it was found necessary for the practical execution of the bactericidal tests to adopt a scheme which would involve the least amount of work and still furnish a satisfactory measure of the activity of the substances. For this reason the drug dilutions, which as a rule started with 1:200, were doubled in each successive dilution so that the series 1:200, 1:400, 1:800, 1:1,600, etc., were the concentrations with which the observations were made. With this scheme it is seen that as the dilutions increase the differences between them become greater, making it possible to regard the figures obtained only as rough approximations to the true values. In the strictest sense account should be taken of the molecular weights of the substances in a direct comparison of their bactericidal properties, but with the dilution scheme here employed this was deemed unnecessary.

In spite of the crudity of our figures it will be seen that certain relationships between the constitution and the bactericidal action are plainly in evidence. The results given below clearly demonstrate that by the addition of hexamethylenetetramine to benzyl chloride a bactericidal substance is obtained, and that by the substitution in the benzene nucleus of different atoms and groups this action may be altered at will, the resulting effect depending upon the number, character, and position of these substituents. In this class of compounds we possess a new group of bactericidal substances in which the hexamethylenetetramine nucleus is directly responsible for their bactericidal character.

#### EXPERIMENTAL PART.<sup>2</sup>

*Technique.*—A strain of *Bacillus typhosus* which had been growing on artificial media for several years and which is a good agglutinator was used in testing the germicidal effects of the compounds.

0.5 or 1 per cent solutions of the substance to be tested were made up in physiological salt solution and filtered immediately through a Berkefeld N filter. With sterile salt solution the dilutions of 1:200, 1:400, 1:800, 1:1,600, 1:3,200, 1:6,400, and 1:12,800 were made and

<sup>2</sup> Some of the tests with *B. typhosus* were performed by Dr. Paul F. Clark, now of the University of Wisconsin, to whom we are greatly indebted for furnishing us with the results of his experiments.



all brought to a temperature of 37°C. To 4 cc. of each dilution, there was added 0.5 cc. of a 24 hour broth culture of *Bacillus typhosus*, and the tubes were put into an incubator or water bath at 37°C. for 4 hours. At the end of this time, one small loopful was taken from each tube and plated in plain agar. It was found necessary to incubate the plates for 48 hours before counting, because some of the colonies did not grow out in 24 hours. Control plates under the same conditions usually contained about 1,000 colonies. Duplicates were run in each case.

Table I presents the bactericidal results obtained with the different preparations tested upon *Bacillus typhosus* by the technique described above. As stated in the introduction, the number of preparations tested and the pressure of other work rendered impossible a detailed study of each substance employed in the tests, so that the figures here given can be regarded only as approximations to the true bactericidal powers of the substances in question. In most instances the figures as given are, if anything, too low. A consideration of the scheme of dilutions employed will show how great the underestimation of the true bactericidal power might be. For example, in the case of the *o*-nitrobenzylhexamethylenetetraminium chloride, given in Table I, the greatest dilution in which this substance killed all the bacteria in 4 hours was 1:3,200. The next higher dilution tried was 1:6,400 and this was found ineffective. But if this compound could really kill in a dilution of 1:5,000 or even 1:6,000, the value obtained as a result of the scheme of dilution used would be only 1:3,200. For this reason we must regard the values given only as relative. In spite of this, the alteration in character or position of the substituents in the nucleus was accompanied by changes in the bactericidal action which were too marked to be masked by the dilution scheme employed.

The tests with hexamethylenetetramine itself and the simple aliphatic quaternary salt methylhexamethylenetetraminium iodide showed them to be devoid of action in a dilution of 1:200. By the substitution in the latter compound of the methyl by the benzyl group the customary influence of the aromatic nucleus was observed. Although not a strong bactericide, the benzyl salt was found to kill all the bacteria present in a dilution of 1:200. This bactericidal

TABLE I.

Substance.	Killed <i>B. typhosus</i> in 4 hrs. at 37°C. in a dilution of 1:
Hexamethylenetetramine.....	+
Methylhexamethylenetetraminium iodide.....	+
Benzylhexamethylenetetraminium chloride.....	200
<i>o</i> -methylbenzylhexamethylenetetraminium chloride.....	3,200
<i>m</i> - " " ".....	800
<i>p</i> - " " ".....	800
3, 5-dimethylbenzylhexamethylenetetraminium chloride.....	400
<i>o</i> -chlorobenzylhexamethylenetetraminium ".....	1,600
<i>p</i> - " " ".....	800
<i>o</i> -bromobenzylhexamethylenetetraminium ".....	1,600
<i>p</i> - " " ".....	200
<i>o</i> -iodobenzylhexamethylenetetraminium bromide.....	1,600
<i>p</i> - " " ".....	1,600
<i>o</i> -cyanobenzylhexamethylenetetraminium chloride.....	3,200
<i>p</i> - " " ".....	400
<i>o</i> -nitrobenzylhexamethylenetetraminium ".....	3,200
<i>m</i> - " " ".....	400
<i>p</i> - " " ".....	1,600
2, 4-dinitrobenzylhexamethylenetetraminium ".....	3,200
<i>o</i> -methoxybenzylhexamethylenetetraminium ".....	+
<i>p</i> - " " ".....	200
2, 3-dimethoxybenzylhexamethylenetetraminium chloride.....	+
3, 4- " " ".....	200
3, 4-methylenedioxybenzylhexamethylenetetraminium chloride.....	200
5-nitro-2-methoxybenzylhexamethylenetetraminium ".....	400
3-nitro-4- " " ".....	800
2-nitro-3, 4-dimethoxybenzylhexamethylenetetraminium ".....	3,200
2-acetoxy-3, 5-dibromobenzylhexamethylenetetraminium bromide.....	1,600
4-acetoxy-3, 5- " " ".....	1,600
2-acetoxy-3, 5-dimethyl-4, 6-dibromobenzylhexamethylenetetraminium bromide.....	1,600
2-acetoxy-3, 5-dimethylbenzylhexamethylenetetraminium chloride.....	+
3-carboxy-4-oxybenzylhexamethylenetetraminium ".....	1,600
3-carbomethoxy-4-oxybenzylhexamethylenetetraminium ".....	400
2-methoxy-5-carboxybenzylhexamethylenetetraminium ".....	400
2-methoxy-5-carbomethoxybenzylhexamethylenetetraminium ".....	400
<i>o</i> -acetaminobenzylhexamethylenetetraminium chloride.....	800
<i>p</i> - " " ".....	+
1, 2-xylylenedi-hexamethylenetetraminium dichloride.....	12,800
1, 3- " " ".....	6,400
Mesitylylenedi-hexamethylenetetraminium ".....	12,800

\* + indicates growth after exposure to a dilution of 1: 200.

power was further developed by the introduction into the nucleus of various atoms and groups, resulting in the series of substances given in Table I. A study of these brings out the following relationships.

The methyl, chlorine, bromine, iodine, cyano, and nitro groups were all found to increase the bactericidal power of the parent unsubstituted benzyl compound. This behavior of the alkyl, halogen, and nitro group has been frequently observed with other types of organic bactericides; for instance, in the case of the phenols. However, this effect may by no means be regarded as inevitable, as there are many bactericidal substances the power of which is in no way influenced by the introduction of these groups. Examples of this will be found among other types of hexamethylenetetraminium salts to be described in the following paper.

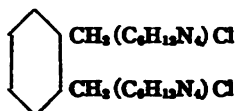
A perusal of the table will show the interesting influence of the position of the substituent upon the bactericidal action. In most cases the *ortho* substituents were found to be more active than either the *meta* or *para* compounds. The regularity of this rule, at least as far as the present series was extended, is striking. The *ortho* methyl, chloro, bromo, nitro, and cyanobenzyl salts were all more active than their other position-isomers.

With this type of hexamethylenetetraminium salt the methoxy group is, on the whole, without influence on the bactericidal effect, if indeed not detrimental. In those compounds which contain the nitro group besides the methoxy group the influence of the former appears to dominate. The 2-nitro-3,4-dimethoxybenzyl derivative was found to equal in effectiveness the 2-nitrobenzylhexamethylenetetraminium chloride. In the same way the 3-nitro-4-methoxy compound was bactericidally about as powerful as the 3-nitrobenzyl salt.

The optimum effect in varying substituents was apparently obtained with the mono-substituted benzyl compounds. In the few cases in which the dimethyl, dibromo, and dinitro derivatives were made, it was found that no advantage as regards the bactericidal value was to be gained, as a rule, by the multiplication of groups. At the same time the greater the number of substituents, particularly in the case of the nitro and halogen compounds, the less was the solubility in water.

In one direction, however, at least when *Bacillus typhosus* was used

as the test object, it was found that a distinct advantage was to be gained by the multiplication of groups; namely, in those compounds in which hexamethylenetetramine was introduced twice into the side-chains. Such salts were obtained by the addition of two molecules of hexamethylenetetramine to  $\omega_1, \omega_2$ -dichloro-*o*-xylene,  $\omega_1, \omega_2$ -dichloro-*m*-xylene, and  $\omega_1, \omega_2$ -dichloromesitylene. These substances possess the following structural formula, in which, of course, the relative positions of the side-chains are different in each case.



These compounds are the strongest bactericides of this group.

Here it should be emphasized that the bactericidal results obtained with this group of substances refer only to their behavior towards *Bacillus typhosus*. As the work developed, other species of bacteria were made the object of an occasional test, but owing to the incompleteness of the results obtained, and to the fact that the technique was varied, it has not been deemed advisable to enlarge on these results in the present paper. It may, however, be said that in general this class of substances was considerably less effective against the streptococcus and meningococcus, but that the results with the gonococcus approached those obtained with *Bacillus typhosus*. This particular group of hexamethylenetetraminium salts cannot, therefore, be regarded as general disinfectants. As a matter of fact, there are but few, if any, organic bactericides which act uniformly against all species or strains of bacteria. A few cases have been selected in Table II to afford a comparison of the effects of several of these salts upon different microorganisms. With the streptococcus, meningococcus, and gonococcus the technique was altered, the time of exposure of the bacteria to the drug being changed to 3 hours and the temperature to 20°. Such a change in technique should, of course, alter the results, but our experience has shown that this rarely exceeded the space of one whole dilution. It is seen from the table that the nature of the substance used determined the effect upon a particular microorganism. A constant relation between the resistances of the various types of organisms is out of the question. The far greater effectiveness

of the two dihexamethylenetetraminium salts against *Bacillus typhosus* is striking. These substances may be classed as "partially specific" for this species.

TABLE II.

Substance.	Killed <i>B. typhosus</i> at 37°C. in 4 hrs. in dilution of 1:	Killed streptococ- cus at 20°C. in 3 hrs. in dilution of 1:	Killed meningococ- cus at 20°C. in 3 hrs. in dilution of 1:	Killed gonococ- cus at 20°C. in 3 hrs. in dilution of 1:
Benzylhexamethylenetetraminium chloride.....	200	+	400	800
<i>p</i> -methylbenzylhexamethylenetetramin- ium chloride.....	800	+		
<i>o</i> -bromobenzylhexamethylenetetramin- ium chloride.....	1,600	+	+	1,600-3,200
<i>o</i> -cyanobenzylhexamethylenetetraminium chloride.....	3,200		400	1,600
<i>p</i> -cyanobenzylhexamethylenetetraminium chloride.....	400		400	800
<i>o</i> -methoxybenzylhexamethylenetetramin- ium chloride.....	+	+	200	800
3, 4-methylenedioxybenzylhexamethyl- enetetraminium chloride.....	200	+	1,600	800
<i>o</i> -nitrobenzylhexamethylenetetraminium chloride.....	3,200	3,200	800	800
<i>m</i> -nitrobenzylhexamethylenetetraminium chloride.....	400		1,600	1,600
2-acetoxy-3, 5-dibromobenzylhexamethyl- enetetraminium bromide.....	1,600	3,200	800	800
2-acetoxy-3, 5-dimethylbenzylhexamethyl- enetetraminium chloride.....	+	1,600	800	800
<i>m</i> -xylylenedihexamethylenetetraminium dichloride.....	6,400	200	+	1,600
Mesitylylenedihexamethylenetetraminium dichloride.....	12,800	+	400	400

\* + indicates growth after exposure to a dilution of 1:200.

From the consideration of the above observations we feel justified in attributing essentially to the hexamethylenetetraminium group the property of determining the bactericidal character of this class of compounds. For direct comparison with another basic side-chain *p*-nitrobenzylpyridinium chloride was prepared. This was found to be ineffective towards *Bacillus typhosus* even in a concentration of 1:200

after 4 hours' contact. The corresponding hexamethylenetetramine quaternary salt killed *Bacillus typhosus* in a dilution of 1:1,600 in 4 hours.

The function, however, of determining the extent and character of this bactericidal property must be attributed to the substituting groups and to the positions occupied by them in the benzene nucleus to which the hexamethylenetetramine is linked. Our experience has shown that such groups likewise decide other biological properties of this class of substances. Without stopping here to deal at length with the toxicity experiments it may be said that in general the toxicity of these compounds is determined by such groups. For example, whereas the *o*-nitro benzyl derivative could be given to mice intravenously in amounts up to 500 mg. per kilo, the 2,3-dimethoxybenzyl derivative was found to be fatal in 0.1 of this dose.

#### SUMMARY.

By the addition of substituted benzyl halides to hexamethylenetetramine, a series of quaternary salts of this base was obtained. These salts represent a new group of organic bactericides. The results obtained in the tests with these substances upon *Bacillus typhosus* have demonstrated the existence of direct relationships between chemical constitution and bactericidal action within the series.

The bactericidal character is directly attributable to the presence of the hexamethylenetetramine nucleus. The degree of the bactericidal action, however, is determined by the position, character, and number of the groups substituted in the benzene nucleus.

By the introduction of the methyl, chlorine, bromine, iodine, cyano, and nitro groups into the benzene nucleus of the parent benzyl hexamethylenetetraminium salt, the bactericidal power of this compound was notably enhanced. The substitution of these groups in the *ortho* position almost invariably resulted in substances which were more active than their *meta* or *para* isomers. The introduction of the methoxy group was without marked effect.

Several substances in which two hexamethylenetetraminium side-chains occurred were found to be the most active of the substances of this series when tested against *Bacillus typhosus*. Comparative tests with other bacterial types demonstrated that these compounds possessed a marked degree of specificity for *Bacillus typhosus*.

## THE BACTERICIDAL PROPERTIES OF THE QUATERNARY SALTS OF HEXAMETHYLENETETRAMINE.

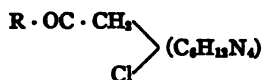
### III. THE RELATION BETWEEN CONSTITUTION AND BACTERICIDAL ACTION IN THE QUATERNARY SALTS OBTAINED FROM HALOGENACETYL COMPOUNDS.

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(Received for publication, March 1, 1916.)

In the course of the biological investigations of the substituted benzyl quaternary salts of hexamethylenetetramine discussed in the previous paper, it was found that the further extension of this chemical type was no longer advisable, in spite of the fact that within this group substances had been found which possessed considerable bactericidal power against *Bacillus typhosus*. This was due partly to the inaccessibility or insolubility of the further preparations of this type which seemed theoretically indicated. Our attention was in consequence turned to the problem of finding a new scheme of chemical procedure which would furnish the possibility of new leads and of broader development. This was found in the readiness with which hexamethylenetetramine as a tertiary base reacted with halogenacetyl derivatives to form soluble quaternary salts with the following general structure,



in which R might represent the radical of any aliphatic or aromatic primary or secondary amine  $\text{R}'\text{NH}$ ,  $\text{R}'_2\text{N}$ ,  $\text{R}'\text{R}'_2\text{N}$ , or of any alcohol or hydrocarbon. Because of the general nature of this reaction and the practically limitless number of chemical possibilities offered, it was found possible to furnish the most varied chemical groupings

with the hexamethylenetetramine molecule by the use of the halogenacetyl side-chain.<sup>1</sup>

Without anticipating the detailed discussion of the observations to be found in the experimental part, the following remarks may be taken to sum up the principal results of the work presented in this communication.

As a result of experiments on a large number of drugs of this type, the general statement may be made that hexamethylenetetramine, when combined with halogenacetyl compounds in the form of quaternary salts, gives rise to a new group of organic bactericides. Hexamethylenetetramine may, therefore, be described as a definite bactericidogenic group. The extent of the bactericidal power of these substances is, in a measure, controlled by the general character of the molecule or of the particular groups contained therein. In addition, the employment of several species of microorganisms has served the purpose of bringing out many examples of partial specificity, at least one substance with a high degree of specificity being found for each of the four species of bacteria used.

The fact that this specificity shifted from one group of bacteria to another with the change in the chemical composition of the radical added to the hexamethylenetetramine, demonstrated that, in contradistinction to the bactericidogenic character, the specificity relations were determined, not by the hexamethylenetetramine nucleus, but by the added radicals.

From the facts set forth above it will be seen that the program presented in the introductory paper has been partially fulfilled. Thus it was found possible, by starting with the molecular grouping furnished by hexamethylenetetramine, to add the most varied atomic groupings with the aid of the  $-\text{CH}_2\text{CO}-$  group, which served merely as a connecting link. The chemical differences in these added groups caused the wide variations observed in the bacteriological results.

In these observations, which must be regarded only as a beginning, we thus see that once in the possession of a biologically active or potentially active molecular group, it is possible, without produc-

<sup>1</sup> For the chemistry of these substances and the reference to those prepared by others see Jacobs, W. A., and Heidelberg, M., *Jour. Biol. Chem.*, 1915, **xx**, 685; 1915, **xxi**, 103, 145, 403, 439, 455, 465.



ing profound chemical changes in the molecule itself, to equip it with a reactive side-chain which in turn will react with other molecular groups and which will furnish the necessary biological properties.

In the present paper we wish to present the results of the bactericidal tests performed with these preparations upon *Bacillus typhosus*, streptococcus, meningococcus, and gonococcus. Here, as in the previous communication, the large number of tests which were made necessitated the early adoption of a rough scheme of drug dilutions. For this reason what was said in the former paper regarding the accuracy of the results must be reiterated here. At best the figures given may be considered as rough approximations. In spite of this, however, in many instances pronounced evidence of the influence of constitution on bactericidal action appeared.

#### EXPERIMENTAL PART.<sup>2</sup>

*Technique.*—In testing the germicidal efficiency of the compounds to be described below, 0.5 or 1 per cent solutions of each substance, according to the solubility, were made in distilled water and filtered through a Berkefeld N filter. The other dilutions were prepared from this stock solution by the use of sterile distilled water as a diluent.

The series 1: 200, 1: 400, 1: 800, 1: 1,600, 1: 3,200, 1: 6,400, and 1: 12,800 was employed for the tests. In some cases the sparing solubility of the substance necessitated the omission of the lower dilutions.

5 cc. of each dilution were placed in wide mouthed tubes and the temperature was brought to 20°C. To each of these tubes the bacterial suspensions were added.

In the case of the *Bacillus typhosus* 0.1 cc. of a 24 hour broth culture was added to each of the tubes containing the dilutions of the compound. After 3 hours a standard 4 mm. loop of the mixture was plated in order to determine the number of living organisms. The plates were incubated for 48 hours before counting. Another loopful from the same tube was taken at practically the same time and inoculated into tubes containing 10 cc. of plain broth and the tubes were

<sup>2</sup> We are greatly indebted to Dr. Martha Wollstein and to Dr. Louise Pearce who conducted the tests with the meningococcus and gonococcus, respectively. Their familiarity with the cultural conditions of the two microorganisms was of special value and service.

incubated for 48 hours. It was found that at a certain dilution, using the plating method, there was a very abrupt falling off in the number of colonies in the plates. This point was always marked in the broth tubes by clear-cut differences in appearance of the incubated tubes. The organisms in the lower dilution produced marked turbidity and in the next higher dilution remained absolutely clear. It was apparent that from 50 to 80 organisms were necessary to inoculate a 10 cc. broth tube, so that anything below this number would not show in this culture media. On the other hand, each organism in the plates produced a colony. Having found at the beginning of the work that this point of abrupt decrease in the number of colonies, using the plate method, came always at the same dilution indicated by no growth in the tubes, the plate method was no longer used on account of the large number of drugs tested.

In the streptococcus tests one or two drops of a 24 hour bouillon culture of an ordinary hemolytic strain of streptococcus were added to each dilution tube. After 3 hours at 20°C. a loopful was taken from the bottom of each tube and plated in plain agar or blood agar. A bacterial control was run in distilled water and plated immediately after mixing and also at the end of the incubation period. By such a control it was possible to estimate the percentage of killing when complete killing did not occur. The plates were incubated at 37°C. for 18 hours and the results recorded.

In the case of the meningococcus the tests were made by Dr. Wollstein. Average 24 hour growths of the microorganism on sheep serum agar were washed off with 2 cc. of sterile distilled water. 0.5 cc. of this well mixed suspension was added to each tube containing the compound dilution. This was allowed to stand for 3 hours at 20°C. Then 0.2 cc. of each tube was planted on sheep serum agar slants. These were incubated for 48 hours and the readings taken. Controls accompanied each experiment.

For the gonococcus tests conducted by Dr. Pearce an adult strain of the organism was employed. 24 hour growths on ascitic veal agar were washed off with 3 to 5 cc. of normal salt solution. The exact amount of salt solution depended upon the profusehness of the growth. The object was to obtain a decidedly cloudy but not milky suspension of the bacteria. 0.5 cc. of this suspension was then added to the tubes containing the substance dilutions and allowed to stand at

20°C. for 3 hours. 0.2 cc. was then pipetted from the bottom of the tubes and planted on an ascitic veal agar slant and incubated for 48 hours. The readings were then taken. Controls were run in each experiment.

In all the above experiments the tests were run in duplicate.

TABLE I.  
3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	<i>B. typhosus</i> killed in dilution of 1:	<i>Streptococcus</i> killed in dilution of 1:	<i>Meningococcus</i> killed in dilution of 1:	<i>Gonococcus</i> killed in dilution of 1:
Chloroacetamide.....	1,600	800-1,600	800	800-1,600
Chloroacetmethylamide.....	1,600	800	3,200	1,600
Chloroacetethylamide.....	800	800-1,600	3,200	6,400
Chloroacetdimethylamide.....	1,600	1,600	800	1,600
Chloroacetdiethylamide.....	1,600	1,600	1,600	1,600
Chloroacetylperidide.....	200	800		
$\beta$ -iodopropionamide.....	+*	+	800	800

\*+ indicates growth after exposure to a dilution of 1:200.

The results obtained with the hexamethylenetetramine quaternary salts prepared from chloroacetamide and the chloroacetyl derivatives of the simpler aliphatic amines will be found in Table I. With but few exceptions these substances were found to kill the organisms used for the test in dilutions of at least 1:1,600 in 3 hours at 20°C. On the whole, but little variation in action, at least of a magnitude which could be detected by the dilution scheme employed, was obtained by the addition of alkyl radicals to the amide nitrogen in the chloroacetamide salt. The exceptions presented by the behavior of the methyl- and ethylamide derivatives toward the meningococcus are worthy of note. With these substances the action was observed to be about four times as great as that of the unsubstituted chloroacetamide salt or its dimethyl derivative. The unusual activity of the compound obtained from chloroacetyl ethylamine against gonococcus is also noteworthy.

When it is considered that substances of purely aliphatic nature are represented in this series, the bactericidal power observed is quite unusual. Formaldehyde, which is considered one of the most powerful of the aliphatic bactericides, when tested by the same tech-

nique was found to kill *Bacillus typhosus* in a maximum dilution of 1:1,200. In addition, the molecular weights of these substances are approximately ten times that of formaldehyde, so that if the comparison were strictly drawn the observed figures should be multiplied by ten. On this basis they far exceed formaldehyde in molecular bactericidal power. The activity of the substances of this group as bactericides is attributable entirely to the presence in them of the hexamethylenetetramine molecule.<sup>3</sup>

The comparative results obtained against *Bacillus typhosus* by the same technique with other aromatic substances which have been regarded in the past as strong organic antiseptics are given in Table II. Unfortunately the tests were restricted to *Bacillus typhosus*. Among the substances given in Table I and in those to follow, many will be found which are as active or more active than any given in this table.

TABLE II.  
3 Hours at 20° C.

Substance.	<i>B. typhosus</i> killed in dilution of 1:
Formaldehyde.....	1,200
Phenol.....	+*
Lysol.....	400
Trichlorophenol.....	800
Tribromo- <i>p</i> -cresol.....	1,600
Tetrabromo- <i>o</i> -cresol.....	1,600
Tribromo- <i>m</i> -xylenol.....	1,600
Gentian violet.....	3,200

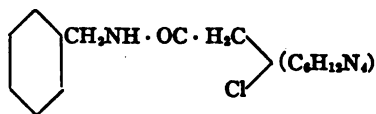
\*+ indicates growth after exposure to a dilution of 1:200.

In an attempt to determine what influence might result from the substitution of the halogenacetic acid radical by that of other halogen fatty acids, it was found that the higher  $\alpha$ -halogen fatty acid

<sup>3</sup> There is evidence that the hexamethylenetetramine molecule does not always persist as such when its quaternary salts are dissolved in water, but undergoes a decomposition which yields methylene derivatives of the corresponding amines. The relation of these substances to the observed effects we shall make the subject of a subsequent communication.

derivatives failed to react with hexamethylenetetramine. The derivatives of  $\beta$ -iodopropionic acid, however, were found to react smoothly with the base to give quaternary salts. The ineffectiveness of the salt obtained from  $\beta$ -iodopropionamide in particular served to indicate the scant promise offered by the further employment of this acid and that the best results would be obtained by the continued use of the halogenacetyl group.

In the logical development of the above substances of purely aliphatic origin, the effect of the introduction of the aromatic nucleus into the alkyl group situated on the amide nitrogen was studied. The opportunity for this was furnished by the quaternary salts obtained from the chloroacetylbenzylamines possessing the following structural formula:



It was thought that here the usual antiseptic influence of the aromatic nucleus would appear, but, as will be seen from the results presented in Table III, this did not conform to the expectations. In interpreting the results, however, the greater molecular weights of these substances should not be neglected. Nevertheless, in those cases in which the observations fell below 1:800 the chemical structure alone must be held responsible. Owing to the irregular character of the fluctuations observed it is difficult to deduce from this table any general relationships between the chemical constitution and the bactericidal power. In the case of the streptococcus and gonococcus, however, the introduction of the methyl group seemed to enhance the activity. The methoxy derivatives also appeared to be more effective than the corresponding acetoxy compounds. It is possible that a series of salts prepared from the mono-substituted benzylamines would have afforded a less confusing and more comparable group of substances for study. The difficulty of procuring such material and the pressure of other work were obstacles to the further extension of this chemical type.

On turning to the more easily accessible chloroacetylanilines, a series of substances was obtained which afforded ample opportunity

TABLE III.  
3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	<i>B. typhus</i> killed in dilution of 1:	<i>Streptococcus</i> killed in dilution of 1:	<i>Meningococcus</i> killed in dilution of 1:	<i>Gonococcus</i> killed in dilution of 1:
Chloroacetylbenzylamine.....	1,600*	800	1,600	800
Chloroacetyl- <i>o</i> -methylbenzyl- amine.....	1,600*	3,200	1,600	3,200-6,400
<i>p</i> -acetaminiodioacetylbenzyl- amine.....	800*	200	1,600	800
1-methyl-4-acetaminochloro- acetylbenzylamine.....	+†	800	800	1,600
1, 2-diacetorychloroacetyl- benzylamine.....	800*	800-1,600	800	400
1, 2-dimethoxychloroacetyl- benzylamine.....	1,600*	800	400	800
1-acetamino-4-ethoxychloro- acetylbenzylamine.....	800-1,600	800-1,600	400	800
$\beta$ -acetoxy- $\alpha$ -naphthochloroacetyl- benzylamine.....	800	1,600	800	800
$\beta$ -methoxy- $\alpha$ -naphthochloro- acetylbenzylamine.....	1,600	3,200	1,600	1,600
<i>m</i> -carbethoxychloroacetylben- zylamine.....	800	400	400	800
<i>m</i> -carbamidochloroacetylben- zylamine.....	+		3,200	1,600
Diethylaminoethyl ester of <i>m</i> - carborychloroacetylbenzyl- amine.....	200	800		

\* Tests in these cases were made at 37° C.

†† indicates growth after exposure to a dilution of 1:200.

for ascertaining the influence of the introduction of groups into the benzene nucleus. These substances possessed the following structural formula, in which any group X may occur in the *ortho*, *meta*, or *para* positions:

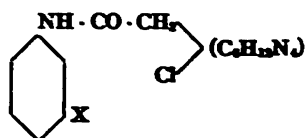


Table IV shows that the linkage of hexamethylenetetramine by means of the chloroacetyl radical with the simpler aromatic amines

TABLE IV.  
3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	<i>B. typhosus</i> killed in dilution of 1:	<i>Streptococcus</i> killed in dilution of 1:	<i>Meningo-</i> <i>coccus</i> killed in dilution of 1:	<i>Gonococcus</i> killed in dilution of 1:
Chloroacetylaniline.....	800	1,600	1,600	3,200
Chloroacetyl- <i>o</i> -toluidine.....	1,600*	1,600	1,600	1,600
Chloroacetyl- <i>p</i> - ".....	800	800	1,600	3,200
Chloroacetyl- <i>m</i> -4-xylydine.....	800	1,600	1,600	3,200
Chloroacetyl- $\psi$ -cumidine.....	800	1,600	3,200	3,200
Chloroacetyl- $\alpha$ -naphthylamine.....	800	800	800	1,600-3,200
Chloroacetyl- $\beta$ - ".....	1,600*	800	6,400	6,400
6-chloroacetylaminquinoline.....	3,200	3,200	1,600	1,600
Chloroacetyl- <i>o</i> -chloroaniline.....	1,600	1,600	800	3,200
Chloroacetyl- <i>p</i> -bromoaniline.....	1,600	1,600	1,600	3,200
Chloroacetyl-5-iodo- <i>o</i> -toluidine.....	1,600	800	1,600	3,200
Chloroacetyl- <i>m</i> -nitraniline.....	3,200	3,200	3,200	3,200
Chloroacetyl- <i>m</i> -nitro- <i>p</i> -toluidine.....	800			
<i>o</i> -chloroacetylaminophenol.....	1,600	800	800	3,200-6,400
Chloroacetyl- <i>o</i> -anisidine.....	800		3,200	1,600
Chloroacetyl- <i>p</i> - ".....	3,200	1,600		
$\beta$ -iodopropionyl- <i>o</i> - ".....	+†	200	400	400
Chloroacetylmethylaniline.....		1,600	800	1,600
Chloroacetyldiphenylamine.....	400	200	1,600	1,600
<i>p</i> -chloroacetylaminobenzoic ethyl ester	1,600	3,200	1,600	3,200
Chloroacetylnovocaine.....			1,600	3,200-6,400
<i>o</i> -chloroacetylaminobenzyl alcohol...	800	800-1,600	800	800
<i>o</i> - " " benzoate...	800	3,200	1,600	
<i>o</i> -chloroacetylaminophenyl "...	200	1,600	800	3,200
<i>o</i> - " " <i>p</i> -nitro-				
benzoate.....	800	1,600-3,200	1,600	1,600
<i>m</i> -chloroacetylaminoacetophenone....		1,600-3,200	800	1,600

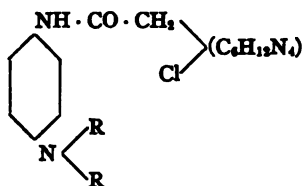
\* Tests were made at 37° C.

† + indicates growth after exposure to a dilution of 1:200.

produced a group of substances possessing definite bactericidal properties. Contrary to the results obtained with the substituted benzyl compounds described in the previous communication, it was found that alterations in the benzene nucleus by the usual substituents did not result in sharp differences in the bactericidal effect, at least of a magnitude which could be revealed by the scheme of dilutions employed. Many instances are to be found, however, in which the ac-

tivity of the simplest member, the salt obtained from chloroacetyl-aniline, has been definitely improved. Among these may be mentioned the substances obtained by the introduction of the methyl, chlorine, bromine, iodine, and nitro groups. Such chemical variations, however, did not always result in an improvement. In many cases the bacteria were killed in dilutions of 1: 3,200 or even 1: 6,400. On the whole, of the microorganisms employed, the gonococcus was the least resistant towards the members of this group. In the absence of indications of a more decided character or of greater regularity there was little assurance of obtaining more powerful bactericides by the further use of these substituents.

The results, however, assumed a different character by the adoption of a new type of variation within this group of substances. This consisted in the use of the dialkylamino group as a substituent in the nucleus of the parent chloroacetyl-aniline quaternary salt. These substances were prepared by the reaction of the chloroacetylaminodialkyl anilines with hexamethylenetetramine and possessed the following structure:



in which R may be methyl, ethyl, etc. The bactericidal results are given in Table V.

TABLE V.  
3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	<i>B. typhosus</i> killed in dilution of 1:	<i>Streptococcus</i> killed in dilution of 1:	<i>Meningo- coccus</i> killed in dilution of 1:	<i>Gonococcus</i> killed in dilution of 1:
<i>p</i> -chloroacetylaminodimethylaniline . . . .	+†	800-1,600	1,600	1,600
<i>p</i> -chloroacetylaminodiethylaniline . . . . .		3,200-6,400	1,600	3,200
<i>p</i> -chloroacetylaminodipropylaniline* . . . .		6,400	3,200	6,400
<i>p</i> -chloroacetylaminobenzylethylaniline* . .		6,400	6,400	12,800
<i>m</i> -chloroacetylaminodimethylaniline . . . .		1,600	400	400

\* In these cases, owing to the sparing solubility in water, one mol. of *N* HCl was used to dissolve the substances.

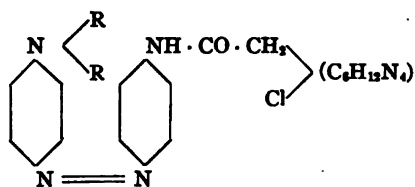
†† indicates growth after exposure to a dilution of 1:200.



Our attention was first called to the desirability of developing this series by the apparent partial specificity of the dimethyl compound for the streptococcus as compared with the effect observed upon *Bacillus typhosus*. Later its effectiveness against the meningococcus and gonococcus was found to be equally marked. The later members of the group were obtained by replacing the methyl groups by ethyl, propyl, and benzyl. In these tests *Bacillus typhosus* was unfortunately omitted. We are therefore in no position to state whether this organism is more resistant to these substances as a class.

The regularity of the response to this particular chemical alteration is strikingly shown by these results. A progressive improvement occurred in the bactericidal action upon all three species of bacteria upon proceeding from the dimethyl to the diethyl, dipropyl, and finally the benzylethyl derivatives, and this in spite of the constant increase in molecular weight. If the increase with each dilution had not been so great, it is probable that all the columns of the table would have shown the regularity of the gonococcus results. The striking feature of the observations is the magnitude of the effect produced by such slight alterations in a complicated molecule. The inferior results obtained with the *meta*-dimethylamino compound, the last in the table, would indicate that the relative positions occupied by the substituents in the nucleus are modifying factors.

The efficacy of these groups was still further demonstrated by their use in another class of substances obtained by the addition of hexamethylenetetramine to chloroacetylaminobenzene derivatives, in which the base was attached by the chloroacetyl amino side-chain to one nucleus and the dialkylamino group to the other as presented in the following formula:



In Table VI the salt obtained from chloroacetylaminobenzotoluene, which contains no dialkylamino group, is first given as an

TABLE VI.  
3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	<i>B. typhosus</i> killed in dilution of 1:	Streptococ- cus killed in dilution of 1:	Meningo- coccus killed in dilution of 1:	Gonococcus killed in dilution of 1:
<i>p</i> -chloroacetylaminooazotoluene.....	3,200	3,200	3,200	1,600
<i>p</i> -chloroacetylaminobenzeneazo- <i>p'</i> -dimethyl- aniline*.....	800	12,800	3,200	3,200
<i>p</i> -chloroacetylaminobenzeneazo- <i>p'</i> -diethyl- aniline.....	+†	12,800	1,600	800
<i>p</i> -chloroacetylaminobenzeneazo- <i>p'</i> -dipropyl- aniline*.....		12,800	800	800
<i>p</i> -chloroacetylaminobenzeneazo- <i>p'</i> -benzyl- ethyl-aniline*.....		12,800	1,600	800
Benzeneazo- <i>m</i> -chloroacetylaminophenol†.....		3,200		

\* In these cases 1 mol. of N HCl was employed to dissolve the substance.

† Solution made by the use of 1 mol. N NaOH.

‡+ indicates growth after exposure to a dilution of 1:200.

object for comparison.<sup>4</sup> The action of this substance upon the different species of bacteria was fairly uniform. The introduction, however, of the dimethylamino group into that position in the molecule farthest removed from the location of the hexamethylenetetramine nucleus resulted in a marked difference. But slight alteration, if any, was observed in the meningococcus, a slight improvement towards the gonococcus, and a considerable reduction in the action upon *Bacillus typhosus*. With the streptococcus, however, the change was profound. The action was increased at least fourfold. The efficacy of this type of chemical modification against the streptococcus was still further confirmed by the replacement of the dimethyl group by the diethyl, dipropyl, and benzylethyl groups. These variations produced compounds which, in spite of the increased molecular weight, exhibited a degree of action of the same order. On the other hand, when tested against the other organisms they were found to be bactericidally less active than the dimethyl compound. We have here an interesting instance of specificity for streptococcus.

<sup>4</sup> The simpler chloroacetylaminooazobenzene derivative could not be conveniently used because of its sparing solubility in water.

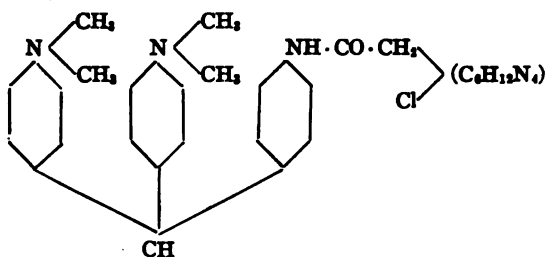
Here again the hexamethylenetetramine molecule was found to be a factor in bringing out this effect. This was directly demonstrated in the following manner: *p*-aminobenzeneazodiethylaniline may be considered as the third substance mentioned in the table deprived of hexamethylenetetramine and the  $-\text{CH}_2\text{CO}-$  radical which serves only as a connecting link. This dye was found to kill the streptococcus in a maximum dilution of 1:3,200, an effect which, though marked, is still but one-fourth of the result obtained with its hexamethylenetetraminium salt.

TABLE VII.  
3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	<i>B. typhosus</i> killed in dilution of 1:	Streptococcus killed in dilution of 1:	Meningo- coccus killed in dilution of 1:	Gonococcus killed in dilution of 1:
<i>p</i> -chloroacetyl-amino- <i>p',p''</i> -tetramethyl- diaminotriphenylmethane ( <i>p</i> -chloro- acetylaminoleukomalachite green)* ....	400	12,800	200	200
<i>o</i> -chloroacetyl-amino- <i>p',p''</i> -tetraethyl- aminotriphenylmethane* .....		12,800	6,400	1,600
<i>p</i> -chloroacetyl-amino- <i>p',p''</i> -tetraethyl- aminotriphenylmethane* .....		51,200	1,600	1,600
Chloroacetyltriphenylamine .....		3,200-6,400	800	800

\* In these cases 1 mol. of N HCl was employed to dissolve the substance.

The group of substances given in Table VII headed by the hexamethylenetetramine quaternary salt of chloroacetyl-*p*-aminoleukomalachite green,

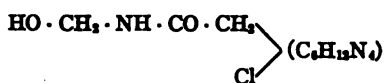


afforded the opportunity of still further testing the value of dialkyl-amino compounds against the streptococcus. This salt, as well as its homologs, displayed a marked specificity for this microorganism, kill-

ing it in a dilution of at least 1: 12,800, whereas the effect on the other forms was relatively weak. In the next two compounds the methyl groups were changed to ethyl groups, and in one case the chloroacetyl-amino radical was shifted to the *ortho* position. The dilution of 1: 12,800 was usually the highest dilution employed for the tests in the routine procedure, but fortunately the experiments performed with the third substance were carried further. This preparation was found to kill the streptococcus even in a dilution of 1: 51,200, making it probable that the first and second substances also would have been found to kill above 1: 12,800.

That here also the hexamethylenetetramine molecule is an essential factor was proven as follows. The first substance given in the table when deprived of this base and its connecting group is nothing else than *p*-aminoleukomalachite green. This substance required a concentration of at least 1: 800 to kill the streptococcus in 3 hours. In other words, the hexamethylenetetraminium salt derived from it was at least sixteen times more active.

In the course of the present work our interest was centered for a time in the study of the biological properties of the hexamethylenetetramine quaternary salts obtained from the chloroacetylalcamines. Our attention was attracted first to this group of substances by the powerful bactericidal properties of the simplest representative, that obtained by the addition of hexamethylenetetramine to chloroacetylaminomethanol.<sup>5</sup>



This substance, which possesses the above structural formula, is the first given in Table VIII. It is seen to possess a marked action against all the species used with the exception of the streptococcus. Because of the unusual effectiveness of this product it was hoped that its suitable chemical variation might lead to a series of very active substances.

<sup>5</sup> This substance was first prepared by Einhorn and Göttler (*Ann. d. Chem.*, 1908, cccxi, 150), who also recognized its antiseptic properties.

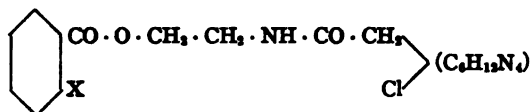
TABLE VIII.  
3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	<i>B. typhosus</i> killed in dilution of 1:	Streptococ- cus killed in dilution of 1:	Meningo- coccus killed in dilution of 1:	Gonococcus killed in dilution of 1:
Chloroacetylaminomethanol.....	3,200	400-800	6,400	6,400
Iodoacetylaminopropanol.....		400	1,600	800
Chloroacetylaminoisopropanol .....		200		
$\delta$ -chloroacetylaminon-butanol.....		400		
$\beta$ -chloroacetylaminon- $\gamma$ - " .....		800		
$\gamma$ -chloroacetylaminon- $\beta$ -methyl- $\beta$ -butanol ....		400		
$\alpha$ -phenyl- $\alpha$ -oxy- $\beta$ -chloroacetylaminopropane...		1,600		1,600
$\beta$ -phenyl- $\beta$ -oxy- $\alpha$ -chloroacetylaminopropane..		800		
Chloroacetylaminopropyl ether.....		1,600		
Chloroacetyl- <i>o</i> -methylphenoxethylamine ...		800		

The chemical development of this substance was attempted in two ways: first, by the replacement of its methanol group by the ethanol, propanol, butanol, etc., radicals; and second, by the acylation of the methanol hydroxyl group with various acid radicals. In the latter scheme, however, chemical difficulties were encountered which compelled the use of its homologs, in particular the ethanol derivative, as the basis for the study of the effect of acylation.

Table VIII presents the behavior principally towards streptococcus of the substances obtained by the first method of chemical variation. The results show that the first member of the group, the salt obtained from chloroacetylaminomethanol, is the most powerful, so that no bactericidal increase was to be gained by such a chemical procedure.

Quite a different result was obtained by the use of the second scheme of chemical variation, as will be seen in Table IX. Unfortunately the inaccessibility of the acylated methanol derivatives made impossible a direct comparison of the effect of acylation upon the chloroacetylaminomethanol salt itself. The results must therefore be referred to the parent unacylated alcamine compound in question for a strict comparison. The structural formula of this group of substances may be represented as follows, X being any substituting group:



A glance at the table will show that we have here another group of hexamethylenetetraminium salts with strong bactericidal properties surpassing in this respect the parent chloroacetylalcamine compound

TABLE IX.

3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	Streptococcus killed in dilution of 1:	Meningococcus killed in dilution of 1:	Gonococcus killed in dilution of 1:
Chloroacetylaminooethyl benzoate.....	800	400	1,600-3,200
“ <i>o</i> -methylbenzoate. . .	1,600	3,200	1,600
“ <i>p</i> - “ . . .	3,200	1,600	800
“ $\beta$ -naphthoate. . . . .	3,200	6,400	6,400
“ <i>o</i> -nitrobenzoate. . . .	800	3,200	1,600
“ <i>p</i> - “ . . . . .	6,400	6,400	3,200
“ <i>p</i> -methoxybenzoate. .	3,200		
“ acetylsalicylate. . . . .	800		
“ <i>p</i> -diethylaminoben- zeneazo- <i>p</i> '-carboxylate . . . . .	6,400-12,800	400	400
Chloroacetyl-amino- $\gamma$ -propyl <i>p</i> -nitrobenzoate .	1,600		
“ $\gamma$ - “ <i>p</i> -methoxyben- zoate. . . . .	1,600-3,200	800	1,600-3,200
Chloroacetylethylaminooethyl <i>p</i> -nitrobenzoate	3,200		
Chloroacetylphenylaminooethyl <i>p</i> - “	800		3,200-6,400

By the introduction of the simplest aromatic acid, benzoic acid, the bactericidal power of the parent iodoacetylaminooethanol salt was doubled, except for the meningococcus. The use of the substituted benzoic acids, such as the methyl, nitro, and methoxybenzoic, and naphthoic acids, in most cases still further improved the action. In the case of the nitrobenzoates the *para* compound seemed more effective than its *ortho* isomer. With the methylbenzoyl derivatives the *para* compound was also more active towards the streptococcus than its *ortho* isomer. With meningococcus and gonococcus the reverse was the case. The specificity of the *p*-diethylaminobenzeneazo-*p*'-carboxylate for streptococcus was to be expected from the results already discussed in connection with Table VI. This substance possesses the

diethylamino group. With the few acids studied, the best results were obtained with the *p*-nitrobenzoyl and  $\beta$ -naphthoyl compounds. The results yielded by the use of other alcamines, such as aminopropanol, ethylaminoethanol, etc., would seem to indicate that the optimum effect is to be obtained with the aminoethanol series.

In this group of substances but a few representatives were made and tested. By the use of numerous other acids a much broader series might be developed for study with the possibility of obtaining more active preparations. However, the observations obtained with this small group of substances serve to demonstrate again to what extent the bactericidal effect may be altered by relatively small changes in the molecule. Here, as in the case of the benzylhexamethylenetetraminium salts discussed in the previous communication, the degree of action is determined by the character and position of the substituents in the benzene nucleus. The main source of the bactericidal effect, however, is still the hexamethylenetetramine molecule.

TABLE X.  
3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	<i>B. typhosus</i> killed in dilution of 1:	<i>Streptococcus</i> killed in dilution of 1:	Meningo- coccus killed in dilution of 1:	<i>Gonococcus</i> killed in dilution of 1:
Chloroacetylurea .....	3,200	800-1,600	1,600	1,600
$\alpha$ -chloroacetyl- $\beta$ -methylurea .....	800	400-800	1,600	1,600
$\alpha$ -chloroacetyl- $\beta$ -benzylurea .....	400	400	800	1,600
Chloroacetylurethane .....	400		1,600	800

Still another type of hexamethylenetetramine quaternary salt included in the investigations was that represented by the compound obtained by the reaction of chloroacetylurea with hexamethylenetetramine. In Table X it is seen that for a purely aliphatic substance it exhibited a strong bactericidal power. It was hoped that by turning to the substituted ureas this action might be improved. The experience with the methyl and benzyl compounds, however, showed only a diminution of the activity.

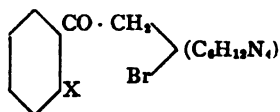
Up to this point the substances which have been the subject of discussion were all quaternary salts obtained from halogenacetyl amino compounds. Two other types of substances were included in the

TABLE XI.  
3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	<i>B. typhosus</i> killed in dilution of 1:	<i>Streptococcus</i> killed in dilution of 1:	Meningo- coccus killed in dilution of 1:	Gonococcus killed in dilution of 1:
Chloroacetone.....	1,600*			
<i>o</i> -bromoacetophenone.....	1,600*	3,200	1,600	1,600
<i>p</i> -methyl- <i>o</i> -bromoacetophenone.....	1,600	800	800	1,600
<i>p</i> -ethyl- <i>o</i> - ".....	800			
1, 2-dimethyl- <i>o</i> - ".....	3,200	3,200	3,200	6,400
1, 3-dimethyl- <i>o</i> - ".....	1,600		800	1,600
<i>m</i> -nitro- <i>o</i> - ".....		1,600		
<i>p</i> -methoxy- <i>o</i> - ".....	800	800	3,200	6,400
<i>p</i> -ethoxy- <i>o</i> - ".....	800	1,600	1,600	1,600-3,200
<i>p</i> -acetamino- <i>o</i> - ".....	800	3,200	1,600	3,200
3-acetamino-4-methyl- <i>o</i> - ".....			800	800
3-acetamino-4-tolyl <i>o</i> -iodoethyl ketone	800	1,600-3,200	12,800	12,800
1, 2-diacetoxy- <i>o</i> -iodoacetophenone....	400	1,600	800	800
$\beta$ -[ <i>o</i> -bromoaceto]-quinaldine. ....	200	3,200	3,200	3,200

\* Tests were made at 37° C.

study in which hexamethylenetetramine was joined by means of the halogenacetyl group first to hydrocarbons and then to alcohols. The first of these groups, which was prepared by the addition of halogen ketones to hexamethylenetetramine, may be represented by the following formula:



The results of the experiments made with these substances are contained in Table XI. The bactericidogenic property of hexamethylenetetramine was again demonstrated. The first member, the salt obtained from chloroacetone, was found to kill *Bacillus typhosus* in a dilution of 1:1,600, which is again striking for an aliphatic substance. Among the aromatic representatives the majority killed one or another of the species tested in dilutions of 1:1,600 or more. The behavior of the 1,2-dimethyl-*o*-bromoacetophenone and *p*-methoxy-*o*-bromoacetophenone derivatives toward the gonococcus and the action of the salt obtained from 3-acetamino-4-tolyl *o*-iodoethyl



ketone on the gonococcus and the meningococcus are worthy of note. It is seen that the chemical constitution of the compounds determines in a degree the bactericidal power, but any definite regularity is far from apparent. As in many instances to be seen in the other tables, the result of a particular chemical variation upon the bactericidal power varies according to the organism used for the test. An interesting instance of the influence of the relative positions occupied by substituents in the benzene nucleus upon the bactericidal effect is shown by the differing action of the 1,2- and the 1,3-dimethyl- $\omega$ -bromoacetophenone salts towards the meningococcus and the gonococcus. The former substance is four times more active than the latter.

The results yielded by the salts obtained from halogenacetyl esters are given in Table XII. The ease of saponification of this chemical

TABLE XII.  
3 Hours at 20° C.

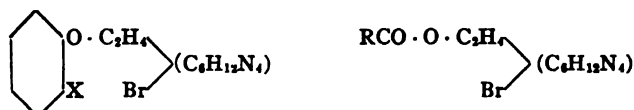
Hexamethylenetetramine quaternary salt of	<i>B. typhosus</i> killed in dilution of 1:	<i>Streptococcus</i> killed in dilution of 1:	Meningo- coccus killed in dilution of 1:	Gonococcus killed in dilution of 1:
Ethyl bromoacetate.....	400	1,600	800	800
Phenyl ".....	800	3,200	1,600	3,200
Bornyl ".....	3,200			
Menthyl ".....	800	1,600-3,200	1,600	1,600
Ethyl $\beta$ -iodopropionate.....	+	200	200	400
Anisoylglycol chloroacetate.....	1,600	1,600	1,600	1,600
<i>p</i> -nitrobenzoylaminoisopropyl chloroac- etate.....	1,600	1,600		1,600

\*+ indicates growth after exposure to a dilution of 1:200.

type limited its more extended development. The table demonstrates the bactericidogenic properties of hexamethylenetetramine in this combination also. The relatively low bactericidal power of the  $\beta$ -iodopropionyl derivative is also in line with the results obtained with other derivatives of this acid.

In the course of the work still other connecting groups than the halogenacetyl radical were used in order to combine hexamethylenetetramine in the form of quaternary salts with other molecular group-

ings. Bromoethyl alcohol by virtue of its alcoholic hydroxyl group may combine with acids to form bromoethyl esters or may be considered the mother-substance of the bromoethyl ethers. These bromoethyl derivatives react readily with hexamethylenetetramine, giving the two following classes of salts:



The results obtained with the first of these, the bromoethyl ether salts, are given in Table XIII. It is to be observed that this type was, on the whole, most active against the meningococcus and the

TABLE XIII.  
3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	<i>B. typhosus</i> killed in dilution of 1:	<i>Streptococcus</i> killed in dilution of 1:	Meningo- coccus killed in dilution of 1:	Gonococcus killed in dilution of 1:
Phenyl bromoethyl ether.....	200	+	400	800
<i>o</i> -methylphenyl bromoethyl ether....	+*	400	1,600	1,600-3,200
<i>m</i> - " " " ".....	400		1,600	1,600
<i>p</i> - " " " ".....	400		400	800
$\alpha$ -naphthyl bromoethyl ether.....	+	800	3,200	12,800
$\beta$ - " " " ".....	800	1,600-3,200	1,600	3,200-6,400
<i>p</i> -bromophenyl " ".....	200	+	3,200	1,600
Tribromo- <i>p</i> -cresyl " ".....		3,200	800	800
<i>o</i> -acetaminophenyl " ".....			400	400
<i>p</i> - " " " ".....			200	200

\*+ indicates growth after exposure to a dilution of 1:200.

gonococcus. The partial specificity of the  $\alpha$ - and  $\beta$ -naphthol bromoethyl ether salts for the gonococcus is especially noteworthy. The  $\alpha$ -compound, which killed the gonococcus in a dilution of 1:12,800, was ineffective against *Bacillus typhosus* in a concentration of 1:200. These instances, together with the other substances mentioned in the table which were found to kill one or the other microorganism in dilutions of 1:1,600 or 1:3,200, still further indicate how general in character is the bactericidogenic property of the hexamethylenetetramine molecule.

TABLE XIV.  
3 Hours at 20° C.

Hexamethylenetetramine quaternary salt of	<i>B. typhosus</i> killed in dilution of 1:	Streptococ- cus killed in dilution of 1:	Meningo- coccus killed in dilution of 1:	Gonococcus killed in dilution of 1:
Bromoethyl acetate.....	+		800	400
“ benzoate.....	200	400	400	800
“ <i>p</i> -nitrobenzoate.....	+		800	800
Bromoethylphthalimide .....	+	+	400	800

\* + indicates growth after exposure to a dilution of 1:200.

In the case of the bromoethyl ester salts (Table XIV) the introduction of the hexamethylenetetramine molecule is seen to be considerably less effective. However, here again the best results were obtained with the meningococcus and the gonococcus. The last substance in the table is not an ester but a bromoethylamino compound. This also was most active against the meningococcus and gonococcus. It would seem from both this series and the previous group of substances that there is something in the chemical nature of the salts obtained from bromoethyl compounds which renders them most active against these two species of bacteria. Although not as marked, this may be considered analogous to the specific effect of the dialkylamino group upon the streptococcus.

It is highly probable that the further development of any of the leads which have been indicated in these papers might eventually furnish more active preparations which would be of chemotherapeutic value.

In conclusion we wish to present the results obtained in a few experiments on the effect of serum and protein on the bactericidal action of several of the compounds mentioned in the preceding tables. It so happened that in these serum-compatibility tests the technique employed was that described in the preceding paper. For this reason the parallel observations made with solutions of the compounds in physiological salt solution are a dilution higher than those to be found in the preceding tables. Table XV presents the results of these tests.

TABLE XV.

Hexamethylenetetramine quaternary salt of	<i>B. typhosus</i> killed in 4 hrs. at 37° in a dilution of 1:	
	In physiological salt solution.	In horse serum.
Chloroacetyl- <i>o</i> -toluidine.....	3,200	3,200
Chloroacetylaminomethanol.....	6,400	3,200
$\omega$ -bromoacetophenone.....	1,600	800
1, 2-diacetoxychloroacetylbenzylamine.....	1,600	800

It is seen that in the case of the salt obtained from chloroacetyl-*o*-toluidine the action was not inhibited by serum. In the other cases the observed effect was reduced by half in the presence of serum. It is possible that in these cases the apparent inhibition was accentuated by the dilution scheme employed, and that in reality but little relative inhibition occurred. With a few other compounds of this class tested by a different technique a much greater relative inhibition of the bactericidal action was observed. From these experiments we may at any rate conclude that the bactericidogenic hexamethylenetetramine portion of the molecule does not in itself cause serum-incompatibility. The source of this must be sought in the remainder of the molecule.

TABLE XVI.

Hexamethylenetetramine quaternary salt of	Gonococcus killed in 2 hrs. at 20° in a dilution of 1:	
	In aqueous solution.	In 5 per cent so- dium caseinate solution.
Chloroacetyl- $\beta$ -naphthylamine.....	3,200	1,600
<i>p</i> -methoxy- $\omega$ -bromoacetophenone.....	6,400	6,400
Chloroacetyl novocaine.....	6,400	6,400
$\alpha$ -naphthyl bromoethyl ether.....	12,800	6,400
Choloroacetyl aminoethyl- <i>p</i> -nitrobenzoate.....	3,200	3,200
3-acetamino-4-tolyl $\omega$ -iodoethyl ketone.....	12,800	1,600

In Table XVI will be found the results of a series of tests in which the substances were dissolved in a 5 per cent sodium caseinate solution. The gonococcus was here used and the technique employed was the same as that described in the other gonococcus tests. In only one

case, the last given in the table, was any marked inhibition to be observed. With the remaining substances mentioned relatively little or no inhibition was observed.

#### SUMMARY.

The extension of the study of the quaternary salts of hexamethylenetetramine to those obtained by the addition of this base to the most varied types of substances containing aliphatically bound halogen has demonstrated that the introduction of the hexamethylenetetramine nucleus in this manner results in the production of bactericidal substances or enhances the bactericidal action if already present.

In particular it was found possible by the use of the halogenacetyl group,  $XCH_2CO$ , as a connecting link, to furnish primary and secondary aliphatic and aromatic amines, alcohols, and hydrocarbons of the most varied character with the hexamethylenetetramine molecule and to study the relation between chemical constitution and bactericidal action in the series of substances so prepared. Because of the variety of chemical types studied, the results are too involved for a detailed summary here.

Many of the substances were found to be very powerful bactericides, and in a number of instances derivatives of purely aliphatic nature were found to possess an unusual bactericidal power.

*Bacillus typhosus*, streptococcus, meningococcus, and gonococcus were the microorganisms used for the tests, and striking instances of partial specificity were observed. This specificity was found to favor not one species alone, but instances were found in which each of the types of bacilli was shown to be especially susceptible to one or another of the particular types of compound employed. The source of this partial specificity is to be sought not in the hexamethylenetetramine nucleus itself but in the molecule to which it is attached.

The action of some of the substances was tested in the presence of serum or protein and was found to be not at all or only slightly inhibited. In other cases marked inhibition occurred. The factors controlling the serum- or protein-compatibility of these substances are likewise to be sought in that portion of the molecule other than the hexamethylenetetramine.



## THE PROTECTION OF PATHOGENIC MICROORGANISMS BY LIVING TISSUE CELLS.

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PLATE 90.

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The discovery by Metchnikoff of the purposeful character of phagocytosis has so stimulated investigation of the defensive activities of tissue cells that phagocytosis and bacterial destruction are at present almost synonymous in the general mind. The old view that leukocytes provide ingested organisms with a culture medium and a means of transport is now mentioned only in historical résumés. It is known that bacteria may be ingested alive—Metchnikoff himself utilizes this fact in his demonstration of the importance of cells for immunity<sup>1</sup>—and it is known also that a cell may take up too many microorganisms and dying of a surfeit, as one might say, may fail to kill them. But such occurrences are regarded as mere incidents in the process of destruction. The possibility that in certain instances cells not only fail to kill the organisms they ingest, but actively protect them from circulating antibodies seems not to have been considered. Yet the question thus raised has more than passing interest. There are a number of important diseases, among them leprosy, tuberculosis, gonorrhea, Leishmania, caused by microbic parasites which live more or less habitually within tissue cells. The part played by the host cells in the life of such microorganisms and also in the distribution within the body of the diseases they induce has obvious importance.

Unfortunately it is not possible to make direct *in vitro* tests with the microorganisms mentioned and the cells in which they live, for the reason that they fail to give rise to circulating antibodies active

<sup>1</sup> Metchnikoff, E., in Kolle, W., and von Wassermann, A., *Handbuch der pathogenen Mikroorganismen*, Jena, 2nd edition, 1913, ii, 679.

enough to be suitable for the tests. But the problem can be approached by means of artificial systems. It is possible, for example, to submit leukocytes that have ingested bacteria to a bactericidal serum and observe its effect on the intracellular organisms.

*Protection against a Foreign Antiserum.*

In a first experiment we have used *Bacillus typhosus*, the leukocytes of the guinea pig, which, according to Pettersson,<sup>2</sup> contain no substances destructive to typhoid bacilli, and the serum of normal rabbits, which is strongly bactericidal for the organism.

*Experiment 1.*—Washed leukocytes from two sterile, 18 hour, aleuronat exudates of the guinea pig's peritoneal cavity were made into a single thick suspension with Locke's solution, and incubated with typhoid bacilli in the presence of a much diluted mixture of fresh guinea pig serum and antityphoid rabbit serum. The smallest amount of the mixture that would ensure good phagocytosis had been previously determined. Twice this amount was employed. The suspension of typhoid bacilli consisted of four 24 hour slant agar cultures of different strains<sup>3</sup> made up in 80 cc. of Locke's solution.

After 1 hour's incubation films from the phagocytic mixture and from a control mixture without serum were examined, with Manson's stain. Only in the former was phagocytosis observed. It was profuse, though there were still many free bacteria. Now small portions of the mixtures and of other control mixtures were added to large amounts of fresh, normal rabbit serum, and the incubation was continued 2 hours longer. Plating was then done in equal portions of agar. Duplicate tests were made throughout. For the dilutions Locke's solution was used.

As the experiment shows, leukocytes can protect ingested bacilli from the action of a bactericidal antiserum.

The mixture of leukocytes and bacteria subjected to preliminary incubation without serum (Mixture 2), and consequently free of phagocytosis at the end of the first hour, gave many more colonies in the plates made 2 hours after the addition of rabbit serum than did Mixtures 3 and 4 from which leukocytes were absent. This was probably due to the protection of ingested organisms, despite the fact

<sup>2</sup> Pettersson, A., *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1905, xxxix, 423.

<sup>3</sup> These were laboratory strains known respectively as Board of Health, Metchnikoff, Wassermann, and New York Hospital, which had been under cultivation for more than 2 years.



TABLE I.

Mixture.	Locke's solution.	Leukocytes.	Bacteria.	50 % guinea pig serum.	2 % antityphoid rabbit serum.	A.	Colonies per square cm. after 17 hrs.	B.	Colonies per square cm. after 17 hrs.
1	α.	α.	α.	α.	α.	1 hr.'s incubation 0.3 cc. from each tube was mixed with 2.4 cc. normal rabbit serum; and after 2 hrs.' more incubation 0.5 cc. of this mixture was plated in 6 cc. agar.	211 294	Same as A except that 0.3 cc. was mixed with 2.4 cc. Locke's solution instead of serum.	About 500.
2	0.2	0.2	0.2	—	—		72 49		Innumerable.
3	0.2	—	0.2	0.1	0.1		10 13		Exceedingly numerous.
4	0.4	—	0.2	—	—		25 27		Innumerable.

that the bacteria were all outside the cells at the time the rabbit serum was added. For the rabbit serum itself was able to cause phagocytosis and did actually cause this in the mixture, as the films show. Presumably it brought about the ingestion of some living bacteria which were then protected from its further action by the cells containing them.

The character of the protection was not determined in this experiment. The results of Table I can be interpreted otherwise than wholly in terms of bactericidal action. The serum was agglutinative; and agglutination can of itself produce a reduction in the number of colonies from a bacterial suspension. The leukocytes might have protected the bacilli merely mechanically against clumping and thus have brought about the results seen in the plates. Obviously, for further work a non-agglutinating bactericidal agent was desirable.

#### *An Indicator of Cell Death.*

The question came up, furthermore, whether the leukocytes exerting a protective influence were alive. Rabbit serum contains an hemolysin for guinea pig erythrocytes, and might well kill the white cells of this species. To solve the point resort was had to tests with

trypan-blue. Evans and Winternitz<sup>4</sup> state that the dye rapidly colors the nuclei of dead cells but does not stain living ones. The following experiment confirms their observation.

*Experiment 2.*—A 4 day aleuronat exudate from the pleural cavity of a dog was washed and suspended in Locke's solution. It contained many large mononuclear cells capable of phagocytizing rat erythrocytes. A part of the suspension was mixed with rat erythrocytes and dog serum, and incubated for 1 hour, after which an equal bulk of a freshly prepared and filtered solution of trypan-blue (0.02 gm. in 2.0 cc. of Locke's solution) was added and the cells forthwith examined. The nuclei of the majority of them failed to stain. None of the many cells that had phagocytized erythrocytes showed nuclear staining.

Other portions of the original suspension were kept in the ice box for several days and then treated in the same way. Most of the cells now failed to take up the rat corpuscles, and most stained promptly with trypan-blue. Among the few which did not stain were those which had just phagocytized rat cells.

The results of this experiment have been borne out by many subsequent observations involving injury to cells of other types, among them the cells liberated from tissue cultures by digestion of the plasma clot with trypsin.<sup>5</sup> Trypan-blue is a prompt and reliable indicator of whether cells are alive or dead.

#### *Protection against an Inorganic Disinfectant.*

The test with trypan-blue showed that rabbit serum is injurious to guinea pig leukocytes subjected to it under the conditions of Experiment 1. At the end of 2 hours' incubation about half the leukocytes were dead, as shown by the nuclear staining, whereas in control specimens incubated in salt solution, they were nearly all alive, very few stained cells being observed. Because of the unfitness of rabbit serum for our work, as thus manifested, it was necessary to find another bactericidal agent, one that would not harm the leukocytes or agglutinate the bacteria. Potassium cyanide proved to have both these qualities.

Clowes<sup>6</sup> was the first to demonstrate the difference in resistance of tissue cells and bacteria to potassium cyanide. As far as we are

<sup>4</sup> Evans, H. M., and Winternitz, M. C., unpublished work, cited by Evans, H. M., and Schulemann, W., *Science*, 1914, xxxix, 443.

<sup>5</sup> Rous, P., and Jones, F. S., *Jour. Exper. Med.*, 1916, xxiii, 549.

<sup>6</sup> Clowes, G. H. A., *Brit. Med. Jour.*, 1906, ii, 1548.

aware, his important observations have not been followed up. He found that tumor cells treated *in vitro* with cyanide in a concentration that killed bacteria remained capable of causing tumors on implantation. Our tests have shown that  $\frac{N}{100}$  potassium cyanide in Locke's solution will destroy typhoid bacilli in heavy suspension while failing to kill guinea pig leukocytes, as shown by the trypan-blue test. Furthermore, the bacilli are not agglutinated. In Experiment 3 advantage has been taken of these facts.

*Experiment 3.*—This experiment closely followed Experiment 1 except that potassium cyanide was substituted for rabbit serum. A watery solution of potassium cyanide, isotonic with 0.95 per cent sodium chloride, was used, diluted with Locke's solution to  $\frac{N}{100}$  concentration.

No phagocytosis was observed in Tube 2 after the preliminary incubation; but it was pronounced in Tube 1, though large numbers of bacilli were still free. The leukocytes were tested with trypan-blue at the time of plating and were found to be, in general, still alive, as proved by the fact that their nuclei failed to stain. In more concentrated potassium cyanide solutions they died early as shown by the stain. Some potassium cyanide was carried over into the agar plates but there it was greatly diluted. Control tests with regard to this point showed that the addition to agar of more than ten times the amount of potassium cyanide present in our plates failed to prevent or even to delay the growth of typhoid organisms.

TABLE II.

Mixture.	Locke's solution.	Leukocytes.	Bacteria.	50 % guinea pig serum.	2 % antityphoid rabbit serum.	A.	Colonies per square cm. after 17 hrs.	B.	Colonies per square cm. after 17 hrs.
1	—	0.2	0.2	0.1	0.1	1 hr.'s incubation then 1.2 cc. $\frac{N}{100}$ potassium cyanide added; 2 hrs.' more incubation and 0.2 cc. plated with 6.0 cc. agar.	144 142	Same as A except that 1.2 cc. Locke's solution added instead of the potassium cyanide.	About 1,500
2	0.2	0.2	0.2	—	—		2 2		Innumerable.
3	0.2	—	0.2	0.1	0.1		0 0		About 1,500
4	0.4	—	0.2	—	—		0 0		Innumerable.

Here there was a marked protection of bacteria by the tissue cells containing them (Table II). The potassium cyanide entirely sterilized the suspensions in which the bacilli were free, whereas in the mixtures in which phagocytosis had occurred, many bacilli survived its action and grew in the plates. One or two colonies developed from Mixture 2, in which there was, supposedly, no phagocytosis because of the absence of serum. But, as is well known, phagocytosis takes place to a slight extent in the absence of serum. The result is readily explained on this basis.

Despite the results of this experiment, the evidence for a protection exerted by living cells specifically is not conclusive. For had the cyanide killed but one phagocyte in every several hundred,—and this may well have happened,—the gross number would be sufficient to permit of an interpretation of the results in terms of a protection exerted by dead phagocytes, not by living ones.

#### *Protection against an Homologous Antiserum.*

A second objection to experiments such as the foregoing is that they have little in common with the conditions in the animal body. We have sought to meet both these difficulties by employing red cells as the test object, placing phagocytes which contain them in an hemolytic antiserum derived from an animal of the sort furnishing the phagocytes. With such material it is easy to follow the fate of the ingested corpuscles and to determine by means of the trypan-blue stain whether the individual phagocytes exerting protection are alive or dead. And if the time element be disregarded, one can with good reason liken the conditions as regards the ingested rat corpuscles to those affecting pathogenic microorganisms existing within cells bathed with a lymph containing antibodies.

We have employed rat red cells allowing large mononuclear cells from an exudate in the dog's pleural cavity to phagocyte them, after which an anti-rat dog serum has been added to the mixture. In order to follow the fate of the ingested red cells it has been necessary to know what changes they would undergo when injured within the phagocytes by the hemolytic serum. For the corpuscles cannot lake as they would when free. Fortunately these changes proved easily

recognizable when several red cells were present side by side in a single phagocyte. They have been described and figured by Levaditi<sup>7</sup> and others, who noted them, as we have done, within phagocytes that had taken red cells out of an hemolytic mixture after the hemolysin had attached itself. Under such circumstances hemolysis goes on within the phagocyte with the result that the included corpuscles melt together, as it were, forming one or more large, orange-yellow hemoglobin-containing globules, which on pressure escape from the cell to dissolve instantly in the surrounding fluid. The contrast between these intracellular globules and red cells that remain intact after ingestion is pronounced (Fig. 1).

*Experiment 4.*—The phagocytes used were mononuclear cells of a 4 day aleuronat exudate in the pleural cavity of a dog. The exudate was washed twice and made into a thick suspension. It contained a very few red cells. The opsonizing serum was derived from the same dog, and so too the 25 per cent suspension of washed dog cells needed for the controls. The serum hemolytic for rat cells came from another dog which received three intravenous injections of rat erythrocytes on successive days and was bled 8 days thereafter. Preliminary examination of the two dogs' blood had shown that they did not agglutinate or hemolyze each other.

The form of the experiment was simple. Rat cells, dog exudate, and a little normal dog serum were mixed together, and, when phagocytosis had taken place, anti-rat dog serum was added to some portions of the mixture, and to others an equal quantity of Locke's solution. Incubation was resumed and from time to time the phagocytosed red cells were observed for evidence of destruction. Two preliminary tests were necessary.

(A) A determination of the least amount of dog serum which would incite to phagocytosis. The dog serum used for its opsonins contained, as is usual, an hemolysin for rat cells. But the test showed that it could be used to incite phagocytosis in an amount far below the one producing visible erythrocytic change.

(B) A test to find out how much anti-rat dog serum was required to hemolyze free rat cells so rapidly that they could not be taken up by dog phagocytes mixed with them. For the anti-rat serum was not only hemolytic but an active stimulant to phagocytosis as well. And, had it been added to the phagocytic mixtures in a quantity which permitted the taking up of cells while hemolysis of them was going on, a proper comparison between the corpuscles in the phagocytes submitted to serum and those submitted to Locke's solution would have been impossible. The results of this test were so interesting that they will be given in detail.

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<sup>7</sup> Levaditi, C., *Ann. de l'Inst. Pasteur*, 1902, xvi, 233.

TABLE III.

Mixture.	25 per cent rat red blood corpuscles.	Dog serum.	Exudate.	Anti-rat serum.	Apparent hemolysis.	Real condition as determined microscopically.
	cc.	cc.	cc.	cc.		
1	0.1	0.1	0.25	0.5	Complete within 5 min.	No phagocytosis. Complete hemolysis.
2	0.1	0.25	0.25	0.25	Complete. (?)	Considerable phagocytosis. All free red blood corpuscles hemolyzed.
3	0.1	0.25	0.25	0.25 of 50% solution.	+++	Profuse red sediment, of phagocytes enormously distended with red cells.
4	0.1	0.25	0.25	0.25 of 25% solution.	++	All free red cells hemolyzed.

Incubation was for 1 hour at 37°C.

Only in the first mixture was there complete hemolysis and in this all the red cells had suffered destruction within the first 5 minutes of incubation (Table III). In the other mixtures the degree of color of the supernatant fluid at the end of an hour indicated incomplete hemolysis as did the profuse red sediment. But these findings were not due to the serum's lack of hemolytic power, for, as the microscope showed, all the red cells remaining free had been hemolyzed. Many though, had been ingested by cells of the exudate, and thus were protected from hemolysis. The abundant red sediment consisted of phagocytes distended with red cells. Some of the phagocytes had extended only the thinnest layer of glassy cytoplasm over the red corpuscles which stood out, quite unhemolyzed, as knobs on their surface. Such corpuscles were evidently protected from the serum by their intracellular situation. But most of the ingested red cells had been much injured and had coalesced into orange-yellow globules (Fig. 1).

The test made it evident that in order to avoid phagocytosis in the presence of the hemolytic serum sufficient of this serum must be added to cause hemolysis of all the red cells within 5 minutes.

Now the main experiment was proceeded to. The following mixtures were made in a number of tubes.

*Mixture 1.*—0.1 cc. 25 per cent suspension of rat red blood corpuscles + 0.5 cc. exudate + 0.5 cc. 25 per cent dog serum.

*Mixture 2.*—0.1 cc. 25 per cent suspension of rat red blood corpuscles + 0.5 cc. exudate + 0.5 cc. 10 per cent dog serum.

At the end of an hour's incubation the mononuclear cells were found to have ingested numbers of apparently unchanged red cells,—from 6 to 25, as a rule.

To some of the duplicate tubes 0.5 cc. anti-rat dog serum was added, to others the same quantity of Locke's solution, and incubation was resumed. At the end of a further hour the preparations were examined for evidence of destruction of the intracellular red cells. None had occurred. The erythrocytes within the mononuclears submitted to anti-rat serum, like those within the phagocytes treated with Locke's solution, were still intact (Fig. 2). But the anti-rat serum had hemolyzed all extracellular erythrocytes and the phagocytes lay in the midst of masses of shadows.

A still more exacting test was carried out, as follows:

*Mixture 3.*—0.1 cc. 25 per cent suspension of rat red blood corpuscles + 0.5 cc. exudate + 0.5 cc. concentrated dog serum. After 1 hour's incubation 0.5 cc. anti-rat serum was added to some of the tubes, an equivalent amount of Locke's solution to others, and incubation continued for 2 hours more. In this instance the amount of serum used for opsonization caused injury to the red cells, of which some coalesced into globules immediately after their ingestion, though the majority remained intact. And now when the phagocytes full of these globules and of more or less injured and vulnerable cells were submitted to anti-rat serum and to Locke's solution, respectively, for 2 hours, no microscopic differences in their contents were observed. The anti-rat serum had been powerless to enhance the breaking-down of the red cells.

The dog red cells present in the exudate were far too few to constitute a source of error in the findings. Nevertheless, a number of control tests were made with a 25 per cent suspension of dog cells instead of rat cells. They were not phagocytized. In the experimental tests proper, the free dog cells were easily distinguished by their failure to agglutinate or hemolyze in the anti-rat serum.

The results of this experiment were clear-cut. The phagocytes protected red cells within them from the action of a powerful homologous antiserum (Figs. 2 and 3).

#### *Protection a Function of the Living Cell.*

The condition of the leukocytes exerting this protective action remained to be determined. Were they perhaps injured by the anti-serum, despite the absence from it of agglutinins and hemolysins? The failure of the serum to penetrate could be explained in this way. Or was the protection a function of the living leukocytes and of living ones only? Tests with the material of Experiment 4 threw light on these points.

*Experiment 5.*—(A) Cells of the ultimate mixtures of Experiment 4 were examined with trypan-blue. The phagocytes which had been incubated with

anti-rat serum and those submitted to Locke's solution alike failed to take the stain. Many of the white cells that had failed to ingest red corpuscles showed nuclear staining.

(B) The following mixture was made up with the ingredients of Experiment 4: 0.1 cc. Locke's solution + 0.5 cc. washed exudate + 0.5 cc. concentrated anti-rat serum + 0.5 cc. dog serum.

After 1 hour's incubation the cells were separated out with the centrifuge and made up as follows:

0.5 cc. treated cells + 0.1 cc. 25 per cent suspension of red blood corpuscles + 0.5 cc. dog serum.

At the end of an hour profuse phagocytosis had taken place, proving that the leukocytes could not have been seriously injured by the antiserum.

(C) Portions of the ultimate mixtures of Experiment 4, in which phagocytosis had occurred, were kept in the ice box at about 2° C. and examined each day. At the end of the first 24 hours the cellular sediment had largely lost its ruddy color. The microscope showed that this was due to diffusion out of the phagocytes of pigment from the ingested red cells. The majority of the leukocytes had now a ground glass appearance. In Mixtures 1 and 2 there could be seen within the phagocytes the intact stromata of red cells from which the hemoglobin had disappeared. In Mixture 3 the stromata were not so clearly visible. In this instance, one will recall, the majority of the red cells were much damaged previous to ingestion. In all the mixtures there were still some phagocytes containing bright red cells, and in Mixture 3 some with orange-yellow globules. Phagocytes containing one or two intact cells and the shadows of others were not observed; but the protoplasm of many phagocytes was stained light orange, due to the seeping out of the hemoglobin from ingested red elements. With the trypan-blue test it was found that the cells containing bright erythrocytes or globules regularly failed to stain. So too did the cells tinted light orange. Practically all the other leukocytes underwent an immediate nuclear staining.

After 3 days in the ice box the results were identical except that living phagocytes were now rare.

This experiment proved that the protection exerted by the phagocytes in Experiment 4 was not due to injury, but on the contrary was associated with active cell life. When the phagocytes died they became permeable, allowing a rapid diffusion outwards of the hemoglobin from the ingested erythrocytes, as well as diffusion inwards of the trypan-blue stain. It seems highly probable from these facts, as well as from common knowledge of the differences in permeability between dead and living tissues, that when phagocytes die they must lose largely if not entirely their protective power.



## DISCUSSION.

There are in the literature a number of detached observations which corroborate our findings. Bordet found that "cholera spirilla injected into the blood stream of cholera immune animals are taken up by the leukocytes even before they can be subjected to lysis by the circulating lytic antibodies."<sup>8</sup> And Metchnikoff, Levaditi,<sup>7</sup> Briscoe,<sup>9</sup> and others have shown that red cells injected into the previously immunized animal may be phagocyted before they can hemolyze. But so far as we are aware no attention has been paid to these indications of protection by cells. Such protection had no practical importance in the instances cited because the phagocytes themselves were capable of destroying the organisms they had ingested. In our experiments as well the phagocytes may have possessed this ability. The demonstration of protection by them is not thereby invalidated. For the ability of cells to protect ingested organisms from the action of outside agencies must be considered as entirely distinct from the disposition they ultimately make of these organisms.

It remains to be determined how far the protection of microorganisms by living tissue cells, especially cells incapable of killing the microorganisms, is important in disease processes. The phenomenon may have much to do with the survival in the animal body of organisms such as the leprosy bacillus which is so often found living within cells of the fixed tissues; and it may serve to explain in part the therapeutic difficulties in such instances. It may throw light, furthermore, on the formation of new disease foci at points of injury in individuals of high general resistance. For if an infective agent can be "walled off" from the action of the body fluids by the protoplasm of a single cell containing it, there is no reason why it should not be carried unharmed wherever this cell goes.

## CONCLUSIONS.

1. Living phagocytes are able to protect ingested organisms from the action of destructive substances in the surrounding fluid, and even from a strong homologous antiserum.

<sup>8</sup> See Zinsser, H., *Infection and Resistance*, New York, 1914.

<sup>9</sup> Briscoe, J. C., *Jour. Path. and Bacteriol.*, 1908, xii, 66.

2. There is evidence that the protection by phagocytes is largely if not entirely conditioned on their being alive.

3. These facts should be taken into consideration in the study of diseases caused by infectious agents capable of living within tissue cells.

#### EXPLANATION OF PLATE 90.

FIG. 1. Red cells hemolyzing within phagocytes. There is a coalescence of the cells into globules.  $\times 625$ .

FIG. 2. Intact red cells of the rat within dog phagocytes submitted for 1 hour to a powerful anti-rat dog serum. Many of the red cells appear pale because they are out of focus. The only free erythrocytes that have not been laked are a few dog cells.  $\times 625$ .

FIG. 3. Red cells of the rat still intact within dog phagocytes submitted for 2 hours to a dog serum strong'y hemolytic for rat cells. The shadows of numerous erythrocytes hemolyzed while free are just visible.  $\times 625$ .





# THE EFFECT OF DIGITALIS ON THE NORMAL HUMAN ELECTROCARDIOGRAM, WITH ESPECIAL REFERENCE TO A-V CONDUCTION.

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PLATES 91 TO 94.

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A study of the effect of digitalis on the normal human electrocardiogram was undertaken by us through a desire to throw more light on the significance of the various grades of heart block not infrequently produced in patients by only moderate amounts of digitalis. Little attention has hitherto been paid to the careful electrocardiographic study of the influence of digitalis on A-V conduction in the normal human heart.

Cohn and Fraser<sup>1</sup> in 1913 reported the study with the string galvanometer of twelve patients with normal cardiac rhythm, four of them without heart lesion. Digitalis in doses equivalent to 2 to 4 gm. of the leaves produced changes in A-V conduction in all the patients. A partial or complete return to the original conduction time was always produced by atropine. In 1914 Cohn<sup>2</sup> reported an investigation with digitalis of patients having an early stage of heart disease with normal mechanism. He concludes that "An effect on conduction may be set down as a usual effect of giving the drug, apart from specific preexisting injury." In our experiment with normal active young adults we have come to the same conclusion and have evidence to show that this defect in conduction is practically entirely due to increased tone and irritability of the vagus.

In this investigation five healthy young male adults were studied by us electrocardiographically. The Cambridge model of the Ein-

<sup>1</sup> Cohn, A. E., and Fraser, F. R., *Jour. Pharm. and Exper. Therap.*, 1913-14, v, 512.

<sup>2</sup> Cohn, A. E., *Jour. Am. Med. Assn.*, 1915, lxiv, 463.

TABLE I.  
Table of Measurements of Case 1, Age 29 Years.

	Electrocardiogram.										Heart rate.	Blood pressure.		Subjective sensations.
	Amplitudes (10 <sup>-3</sup> volt).						Systolic.	Diastolic.						
	P <sub>0</sub>	R <sub>1</sub>	S <sub>2</sub>	T <sub>2</sub>	T <sub>1</sub>	T <sub>0</sub>								
Normal (before digitalis).....	Nov. 8	0.164	0.065	0.312	0.1	0.8	0.05	0.15	0.15	0	76	118	78	
After 0.3 gm. digitalis.....	" 10				0.1	0.85	0.05	0.2	0.15	0	71			
" 0.6 " ".....	" 11				0.1	0.85	0.05	0.1	0.15	-Tr.	68	112	64	
" 0.9 " ".....	" 12				0.1	0.8	Tr.	0.1	0.15	-Tr.	68			
" 1.2 " ".....	" 13				0.1	0.85	"	0.1	0.1	0	64	124	72	Headache, anorexia.
" 1.5 " ".....	" 14	0.164			0.1	0.85	"	0.1	0.15	-0.05	69			
" 1.8 " ".....	" 15	0.187			0.1	0.8	0.05	0.1	0.15	-Tr.	64	122	72	
" 2.1 " ".....	" 16	0.186			0.1	0.85	Tr.	-Tr.	0.05	-Tr.	64			Palpitation.
" 2.4 " ".....	" 17	0.186			0.05	0.8	0	+0.05	0.05	+Tr.	71	130	70	"
" 2.7 " ".....	" 18	0.203			0.05	0.85	0	-Tr.	0.05	-0.05	70			" and anorexia.
" 3.0 " ".....	" 19	0.205	0.069	0.317	0.1	0.8	+Tr.	-Tr.	0.05	-0.05	77	124	70	Palpitation and nausea.
1 day after stopping digitalis.....	" 20	0.222	0.072		0.1	0.9	"	+Tr.	0.1	+Tr.	69			Arrhythmia, nausea.
2 days " ".....	" 21	0.185			0.1	0.85	0	-0.05	0.1	Tr.	80			Bigeminy at night.

[illegible]

TABLE II.

Table of Measurements of Case 2, Age 27 Years.

Electrocardiogram.															Blood pressure.		Subjective sensations.	
Amplitudes (10 <sup>-3</sup> volt).															Heart rate	Systolic		Diastolic
		P-Q	Q+R+S	Q to end of T	II	Q <sub>1</sub>	R <sub>4</sub>	S <sub>2</sub>	T <sub>2</sub>	T <sub>1</sub>	T <sub>3</sub>							
1915																		
Normal (before digitalis).....	Nov.	80	0.161	0.078	0.344	0.15	0.1	1.45	Tr.	0.3	0.15	0.15	67	130	70	Palpitation.		
After 0.3 gm. digitalis.....	"	10				0.15	0.05	1.35	"	0.3	0.2	0.1	75					
" 0.6 "	"	11				0.15	0.05	1.35	"	0.25	0.15	0.1	72	122	70			
" 0.9 "	"	12				0.1	0.1	1.35	"	0.15	0.05	0.1	79					
" 1.2 "	"	13				0.15	0.1	1.45	"	0.1	Tr.	{ -0.05 +0.05	87	116	82	Malaise.		
" 1.5 "	"	14	0.161			0.1	0.1	1.4	"	0.1	0.05	0.05	75			Headache, malaise.		
" 1.8 "	"	15	0.173			0.1	0.05	1.3	"	0.05	0.1	-0.05	80	102	70			
" 2.1 "	"	16	0.166			0.15	0.1	1.4	"	0.1	0.1	{ -Tr. +Tr.	73			Malaise.		
" 2.4 "	"	17	0.170			0.1	0.05	1.35	"	{ -Tr. +0.1	0.1	{ -Tr. +Tr.	64	112	70	Headache.		
" 2.5 "	"	18	0.172	0.078	0.310	0.1	0.05	1.35	"	{ -Tr. +0.1	0.05	{ -0.05 +Tr.	66	106	70	"		
1 day after stopping digitalis..	"	19	0.167			0.15	0.05	1.2	0.05	0.1	0.05	{ -Tr. +0.05	84					
3 days "	"	21	0.167			0.1	0.05	1.35	Tr.	0.15	0.1	{ -Tr. +0.05	75					
5 "	"	23	0.172			0.15	0.05	1.4	0	0.2	0.15	0.05	62					
8 "	"	26	0.162			0.1	0.05	1.4	Tr.	0.2	0.1	0.05	75					



12 days after stopping digitalis. . .	Nov. 30			0.15	0.05	0.1	70
19 " " " " " " " " " "	Dec. 7			0.15	0.15	0.15	70
After 2.5 gm. digitalis.							
{ 1 min. after exercise. ....	Nov. 18	0.183	0.082	?	0.2	0.1	1.3-1.6
{ 45 " " atropine. ....	"	180.144	0.081	0.259	0.15	0.1	1.4
Normal.	1916						
{ 1 min. after exercise. ....	Jan. 10	0.152			0.2	0.2	1.6+
{ 45 " " atropine. ....	"	100.137			0.15	0.1	1.5
Normal control. ....	"	100.157					
" " " " " " " " " "	"	180.155					
" " " " " " " " " "	1915						
" " " " " " " " " "	May 10	0.170					

thoven string galvanometer was used with non-polarizable electrodes. Photographic plates containing the three leads of Einthoven were taken before the administration of the drug, at approximately 24 hour intervals during the administration and at intervals of a day or two after the drug was stopped until the electrocardiograms had returned to normal. We have used Caesar and Loretz digitalis leaf in amounts ranging from 2 to 3 gm. at the rate of 0.3 gm. daily. Different amounts of digitalis were used in order to compare the durations of the drug effects. The effects of atropine (0.002 gm. subcutaneously) and exercise (a fast run of about one-quarter of a mile) on the normal and on the digitalized electrocardiograms were studied. Several control records were taken in order to determine the normal range of A-V conduction time in the individuals tested.

Measurements were made by projecting the images on the photographic plates upon a glass screen at a magnification of twenty-five diameters. For this purpose a microphotographic apparatus was used. The electrocardiographic intervals were measured off by calipers on a scale of one-sixtieth of an inch and compared with the measurements of the time intervals. Our maximum error is below 0.01 second. Time intervals of 0.2 second were used instead of smaller intervals, such as 0.04 second, because of the greater accuracy of measurement. In work which one of us did with Lewis<sup>3</sup> on the measurement of P-R intervals in experimental curves it was found that the upstrokes of deflections so often fell upon and were obscured by the time lines separating intervals of 0.04 second that these small intervals were given up and 0.2 second time intervals adopted. Three beats were measured on each plate and their average was recorded in the final tables (Tables I to V).

In addition to the determination of the A-V conduction time as obtained from the P-R or P-Q interval (the latter if a Q is present), the Q-end of S and Q-end of T intervals before and after digitalis have been measured; the digitalis effects on the amplitudes of the electrocardiographic deflections, on the heart rates, on the blood pressures, and on the subjective sensations have been studied. On account of the fact that the tension of the string was not always

<sup>3</sup> Lewis, T., and White, P. D., *Heart*, 1914, v, 335.

accurately adjusted allowance for errors has been made in calculating the curves.

Tables I to V contain the measurements of the A-V conduction times as expressed by the P-Q or P-R intervals, the measurements of the Q-end of S and the Q-end of T intervals, the amplitudes of the deflections, the heart rates, the blood pressures, and the subjective sensations. Table VI contains the effects of exercise on A-V conduction in the normal electrocardiogram. Figs. 1 and 2 show the control electrocardiograms of the five subjects and those taken at the end of the digitalis administration. Fig. 3 shows the atropine and exercise effects on the digitalized electrocardiograms. Figs. 4 and 5 illustrate the phenomenon resulting from digitalis in one of the subjects.

#### DISCUSSION.

##### *A-V Conduction.*

Digitalis caused a delay in A-V conduction in four of our five subjects. In three the lengthening was but slight and hardly greater than the normal range of conduction time in these same individuals (Tables II, III, and IV). In none of these three did the P-R interval equal or exceed 0.2 second. In the first subject (Table I) prolongation of conduction time up to 0.3 second occurred after 3.0 gm. of digitalis, but in no one of the five did the P-R interval increase to as much as 0.2 second after the ingestion of 2.5 gm. or less of digitalis. In every instance after digitalis even when the delay in conduction amounted to more than 0.05 second atropine reduced the P-R interval to less than its original value. The increased vagal action occurring with the rapidly slowing pulse after exercise added to the defect already present after digitalis. Normally we have found that immediately after exercise the A-V conduction time is markedly decreased, more even than it is decreased normally by atropine, 0.002 gm. subcutaneously (Tables I, II, and VI). Our maximum shortening was from 0.158 to 0.112 second. Shortening of the P-R interval after exercise was found by Lewis and Cotton<sup>4</sup> in 1913.

<sup>4</sup> Lewis, T., and Cotton, T. J., *Jour. Physiol.*, 1913, xlv, p. lx.

TABLE III.  
*Table of Measurements of Case 3, Age 28 Years.*

Electrocardiogram.										Blood pressure.		Heart n	Subjective sensations.
Amplitude					Systolic		Diastolic						
P <sub>1</sub>	Q <sub>1</sub>	R <sub>1</sub>	S <sub>1</sub>	T <sub>1</sub>	T <sub>2</sub>	T <sub>3</sub>	T <sub>4</sub>	T <sub>5</sub>					
0.05	0	1.1-1.25	0.3	0.2	0.2	0.25	-0.05 +Tr.	68	122 70*				
0.1	Tr.	1.25	0.35	0.2	0.2	0.25	-0.05 +Tr.	60					
0.15	0.05	1.45	0.35	0.1	0.25	0.25	-0.1 +0.05	73	125 68				
0.1	Tr.+	1.3	0.35	{ -Tr. +0.05	0.15	0.15	-0.1 +0.05	73					
0.05	0.05	1.3	0.35	0.1	0.15	0.15	-0.1 +Tr.	70	134 78				
0.1	0.05	1.4	0.35	{ -0.05 +0.1	0.15	0.15	-0.1 +Tr.	63					
0.1	0.05	1.3	0.3	{ -0.05+ +0.05	0.15	0.15	-0.1 +Tr.	64	140 62				
0.1	Tr.	1.35	0.3	{ -0.05 +0.1	0.15	0.15	-0.1 +Tr.	70					
0.05	"	1.3	0.3	{ -0.05 +0.05	0.15	0.15	-0.15	57	126 64				
0.1	0.05	1.35	0.35	{ -0.1 +0.05	0.15	0.15	-0.2 +0.1	63					
										Slight nausea.			

[illegible]

\* Blood pressure estimation made Nov. 9, 1915, after 0.1 gm. digitalis.

The U deflection in this subject is unusually pronounced but is unaffected by digitalis. As the T wave flattens it becomes at length level with the U wave.

From the effect of digitalis on A-V conduction in normal hearts it seems to us reasonable to conclude that there is either an abnormal irritability of the vagus or a damage to the conduction tissue itself, if heart block, even a delay in conduction beyond a P-R interval of 0.2 second, occurs in patients after small or moderate amounts of an active preparation of digitalis (for example 1.0 to 2.0 gm. of Caesar and Loretz standardized leaves in 4 to 7 days). If, however, this drug is continued up to and beyond 3.0 gm., a slight defect in A-V conduction then appearing for the first time may be reasonably ascribed to the digitalis and no blame be placed on the conducting tissue.

#### *Arrhythmia.*

The greater action, or at least the less balanced action, of the vagus at night slowed the heart rate in two of the subjects (Cases 3 and 1) below the usual rate, in one the pulse falling as low as 48 to the minute; in the other subject the vagal activity was still further evidenced by the occurrence of blocked auricular premature beats—a phenomenon dependent on delay in conduction time (Figs. 4 and 5). This arrhythmia, the only one produced in any of the subjects, began to appear after 3.0 gm. of digitalis had been taken. As far as we are aware it is the first recorded observation of such a result from digitalis. It consisted of an interruption of the normal rhythm by premature ectopic auricular contractions (almost isoelectric in the electrocardiogram) without ventricular response. Polygrams and electrocardiograms of the phenomenon were obtained with considerable difficulty because of the fact that the irregularity almost always occurred late at night, apparently when the influence of the vagus was greatest and tended to disappear if an attempt was made to obtain graphic records. At times it occurred so often as to produce a bigeminy—two normal beats followed by a premature auricular contraction during and after which there was a pause in the pulse. This irregularity first appeared when the influence of digitalis was at its maximum, as shown by the P-R interval (0.222 second) 1 day after stopping digitalis. It occurred off and on for the following 8 days and nights, but since the 9th day after stopping digitalis (3 months ago) it has not once occurred. The subject had never had premature beats so far as known prior to the ingestion of the

TABLE IV.

Table of Measurements of Case 4, Age 24 Years.

	Electrocardiogram.						Heart rate.	Blood pressure.		Subjective sensations.
	Amplitudes (10 <sup>-3</sup> volt).							Systolic.	Diastolic.	
	Qr	Ra	Sa	Ta	Ti	Ts				
Nov. 90										
Normal (before digitalis).....	0.15	Tr. 0.95-1.0	0.35	0.3	0.2	0.1	85	140	100	
After 0.6 gm. digitalis.....	0.15	" 1.0	0.3-0.35	0.25	0.15-0.2	0.05±	80	144	100	
" 0.9 ".....	0.15	" 1.0-1.05	0.35±	0.2	0.15	0.05	83			Malaise.
" 1.2 ".....	0.1	" 0.95	0.35	0.2	0.15	0.05	80	122	86	" and head-ache.
" 1.5 ".....	0.15	" 1.05-1.1	0.25-0.3	0.2-	0.1+	0.05+	82			Palpitation.
" 1.8 ".....	0.15	" 0.9-0.95	0.3-0.35	0.2-	0.15	0.05-	83	138	90	Dizziness and anorexia.
" 2.0 ".....	0.15	" 1.0-1.05	0.35±	0.25	0.15	0.1	83	128	90	
1 day after stopping digitalis.....	0.1	0 0.95-1.0	0.3	0.2+	0.2	0.05	81			
3 days ".....	0.15	Tr. 0.95	0.3	0.2	0.15	0.05	76			
5 ".....	0.15	" 1.1	0.35	0.25	0.2	0.1	88			
7 ".....	0.15	" 1.05	0.35	0.2	0.1+	0.1-	86			
11 ".....	0.15	" 1.05±	0.3	0.25	0.2	0.05	86			
14 ".....	0.15	0 1.1±	0.3±	0.3	0.2	0.1±	75			
After 2.0 gm. digitalis.										
{ 1 min. after exercise.....	0.25±	1.0	0.65-0.8	0.7	0.2	0.4	140			
" 20 " " atropine.....	0.25	0 0.9-0.95	0.5	0.3	0.15	0.15	138			
Normal control.....	0.1	Tr. 0.95-1.0	0.3	0.3	0.25	0.1	79			
Dec. 3										

Normal (before digitalis) . .  
 After 0.3 gm. digitalis . . .  
 " 0.6 " " . . . .  
 " 0.9 " " " . . . .  
 " 1.2 " " " . . . .  
 " 1.5 " " " . . . .  
 " 1.8 " " " . . . .  
 " 2.0 " " " . . . .

Light ano-  
 rexia,  
 nausea,  
 dizziness.  
 Light ano-  
 rexia,  
 nausea,  
 dizziness.

TABLE V.  
 Table of Measurements of Case 5, Age 32 Years.

Electrocardiogram.	Blood pressure.		Subjective sensations.
	mm.	mm.	
Amplitudes (10-1 volts).			





drug. For these reasons and because of the fact that it was directly dependent on a certain degree of prolongation of the P-R interval (to 0.295–0.300 second) the arrhythmia can be ascribed to digitalis. The subjective sensations of the irregularity were interesting, for the pause following the ectopic auricular beat could always be foretold by the feeling of the premature auricular systole itself consisting of a wave of fulness rising in the neck and throat. The association of mechanical activity of the auricle with the abnormal deflection in the electrocardiogram is clearly shown in the jugular tracing (Fig. 5 b) taken by Dr. O. F. Rogers, Jr. The production of the premature auricular systole by a mechanical stimulus from the contracting ventricle would at present best explain the fact that the R-P interval is much shorter than the P-R interval just preceding and that it varies little if at all in length.

#### *Amplitudes of Electrocardiographic Deflections.*

*T Wave.*—Cohn and Fraser<sup>1</sup> reported in 1913 their observations that the T wave of the human electrocardiogram was inverted in many of their patients who were under the influence of digitalis. More recently Cohn, Fraser, and Jamieson<sup>2</sup> have shown that digitalis given by mouth to patients began to cause a change in shape and amplitude of the T wave as early as 36 to 48 hours after the administration of the drug had begun, the change increasing as the digitalis was continued and persisting for from 5 to 22 days after the drug had been stopped. It is interesting to note that in all five of our entirely normal subjects, as a result of digitalis the T wave was decreased in amplitude in every lead. There seemed to be no direct connection between the effects of digitalis on the conduction time and on the T wave as indicated particularly well by one subject (Table V), who suffered no defect in A-V conduction but who did show a considerable decrease in the amplitude of T (Fig. 1). We have found that the first electrocardiographic evidence of digitalis and, for that matter, the first evidence, of any sort, of digitalis action is the decrease in the amplitude of the T deflection in the case of normal individuals.

<sup>1</sup> Cohn, A. E., Fraser, F. R., and Jamieson, R. A., *Jour. Exper. Med.*, 1915, xxi, 593.

TABLE VI.

*The Effect of Exercise on Normal Electrocardiograms of Cases 1 and 2.*

		P-Q interval.	Heart rate.
Case 1.	Normal (before exercise).....	0.158	75
	$\frac{1}{2}$ min. after exercise.....	0.112	178
	1 " " " .....	0.128	160
	2 " " " .....	0.167	111
	8 " " " .....	0.176	95
Case 2.	Normal (before exercise).....	0.150 =	90
	$\frac{1}{4}$ - $\frac{1}{2}$ min. after exercise.....	0.117	155
	$\frac{1}{4}$ " " " .....	0.132	145
	1 " " " .....	0.144	135
	2 " " " .....	0.150	115
	5 " " " .....	0.153	99
	10 " " " .....	0.149	102
	30 " " " .....	0.156	86

*Effect of Exercise and Atropine on the Digitalized T Deflection.*

The mechanism by which exercise acts on the heart temporarily removed the traces of the digitalis action on the T wave (Fig. 3), while atropine, 0.002 gm. subcutaneously, actually increased the digitalis effect on this deflection (Fig. 3) although the pulse rate was raised about equally by both procedures. Just the opposite action of these two tests was noted on the digitalized P-R interval.

*P<sub>2</sub>, Q<sub>2</sub>, R<sub>2</sub>, and S<sub>2</sub> Deflections.*—These showed no clear-cut changes in amplitude as the result of the digitalis in our subjects.

## SUMMARY.

Digitalis was given by mouth to five normal young male adults in amounts ranging from 2.0 to 3.0 gm. of standardized leaves in the course of 7 to 10 days. The As-Vs interval was prolonged in four of the five subjects, the greatest prolongation occurring in the case of the subject who received the most digitalis and none at all in one who received only 2.0 gm. There was no prolongation to so great an interval as 0.2 second until 2.7 gm. had been taken. The effects of the digitalis on conduction time began 5 to 6 days after the drug had been started and after 1.5 to 1.8 gm. had been taken. The

effects persisted for 1 to 2 weeks after the drug had been stopped. Atropine removed completely the effect of digitalis on A-V conduction. The slowing heart rate after exercise was accompanied by an enhancement of the defect in conduction. The change in conduction through digitalis was therefore almost entirely, if not entirely, due to increase of vagal tone and irritability.

Digitalis did not affect to an appreciable extent the Q-end of S and the Q-end of T intervals. Exercise and atropine both shortened the ventricular complex Q-end of T while the subject was under digitalis.

The amplitude of the T wave, especially in Lead II, was changed within 48 hours after digitalis had been started, a decrease then beginning which became greater as the drug was continued and which persisted until 10 to 19 days after the digitalis had been stopped. The change in the T deflection preceded by several days the change in conduction time. The T wave, therefore, in the normal subject as well as in the patient gives us the earliest indication of digitalis action.

The amplitudes of P, Q, R, and S were not materially influenced by the amounts of digitalis given.

The pulse rate in two subjects became lower than usual at night as the result of the digitalis; otherwise there was no evidence of vagal action on the sino-auricular node. Blood pressure was uninfluenced by the digitalis. Mild subjective sensations occurred in all the subjects during the administration of the drug.

A curious, hitherto undescribed, digitalis arrhythmia consisting of blocked auricular premature beats occurred in one subject after 3.0 gm. of digitalis had been taken.

*Supplementary Note.*—A second series of five healthy young male adults has recently (March, 1916) been studied by one of us electrocardiographically during a course of digitalis—2.6 to 3.3 gm. Caesar and Loretz leaf in 9 to 11 days. (The drug was weaker than that used in the previous investigation.) Three subjects showed slight prolongation of the P-R interval but no bradycardia; the other two subjects, who had no delay in A-V conduction, showed a marked total bradycardia after the digitalis (heart rate of 43 in each in the middle of the forenoon). The T<sub>2</sub> deflection of the electrocardiogram

was more or less flattened by the digitalis in all five subjects. Atropine given subcutaneously at the height of the digitalis action depressed the T wave still further in every instance.

#### EXPLANATION OF PLATES.

In all figures abscissæ equal 0.2 second and ordinates equal  $10^{-4}$  volts.

Where the galvanometer string has been too slack or too tense, the error is seen in the control deflection. The proper correction in the amplitude has been made. Amplitudes have been estimated to 0.05 of a millivolt.

#### PLATE 91.

FIG. 1. Electrocardiograms (Lead II) of Case 5 (*A*), Case 4 (*B*), Case 3 (*C*), and Case 1 (*D*). The left hand column contains control records; right hand column contains records taken during full effect of digitalis. In the center below is a record of Lead II of Case 1 taken 1 day after digitalis had been stopped and showing the variation in the length of the P-R interval.

#### PLATE 92.

FIG. 2. The three electrocardiographic leads of Case 2 before and immediately after the course of 2.5 gm. of digitalis. The change in the T deflection in each lead is evident.

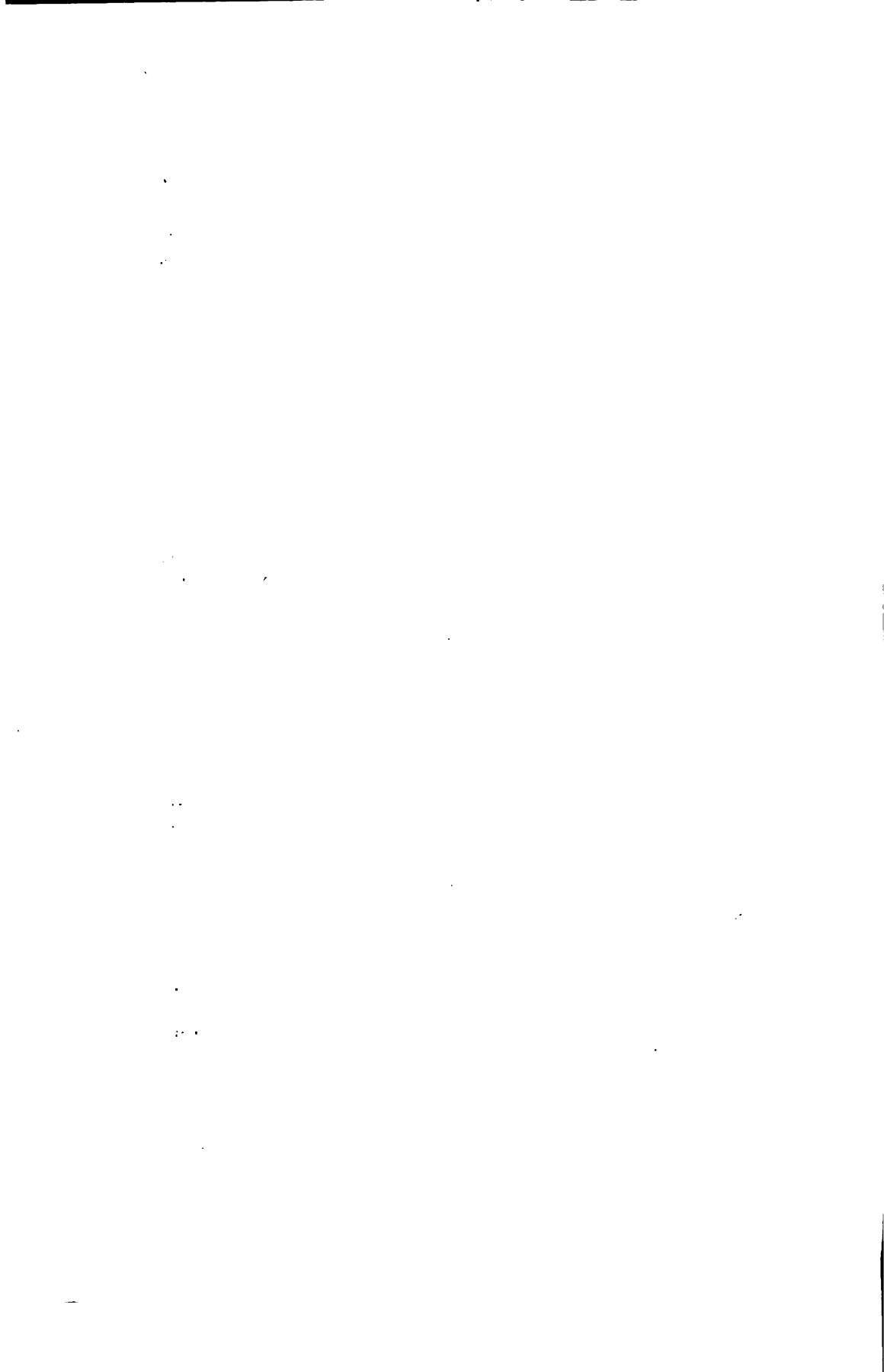
#### PLATE 93.

FIG. 3. Electrocardiograms (Lead II) of Case 4 (*A*), Case 3 (*B*), Case 2 (*C*) and Case 1 (*D*) after atropine (left hand column) and after exercise (right hand column) at the completion of digitalis. In the center below is the record of Lead II of Case 5 after atropine at the completion of 2.0 gm. of digitalis.

#### PLATE 94.

FIG. 4. Lead II of Case 1 showing blocked auricular premature beats following gradual prolongation of the P-R interval. Electrocardiogram taken 5 days after the completion of 3.0 gm. of digitalis.

FIG. 5. Radial pulse tracing (*a*) and polygram (*b*) of Case 1 taken by Dr. O. F. Rogers, Jr., showing arrhythmia produced by the auricular premature beats which occurred as the result of 3.0 gm. of digitalis. *a'*, evidence in jugular pulse of blocked auricular premature beat. Time interval, 0.2 second.



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**A**



**B**



**C**



**D**







THE INFLUENCE OF TETHELIN, AND OF OTHER ALCOHOL-SOLUBLE EXTRACTIVES FROM THE ANTERIOR LOBE OF THE PITUITARY BODY, UPON THE GROWTH OF CARCINOMATA IN RATS.

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*Preparation of the Substances Employed.*

One of us has recently succeeded in isolating the growth-controlling principle (tethelin, from τετηλώς, growing) from the anterior lobe of the pituitary body. The methods of isolating the substance, its chemical properties and physiological actions, and the evidences of its identity with the growth-controlling principle are contained in articles which have been published elsewhere.<sup>1</sup> Briefly summarized, however, the method of isolating the substance is as follows: The anterior lobes of ox pituitaries are carefully separated from the posterior lobes and connecting parts and stripped of their connective tissue capsules. The tissue is then ground up in a mortar with three times its weight of a mixture of equal parts by weight of anhydrous sodium and calcium sulphates. The mixture is then dried on a water bath until nearly white, returned to the mortar, and thoroughly pulverized.

The pulverized material is then extracted with boiling absolute alcohol in an extraction apparatus (of the Bailey-Walker type) so constructed as to carry out the extraction at or near the temperature of boiling alcohol. The extraction is continued for 48 hours. The solution thus obtained is evaporated under reduced pressure until solid material begins to separate out on cooling. To this con-

<sup>1</sup> Robertson, T. B., *Jour. Biol. Chem.*, 1916, xxiv, 397, 409.

centrated solution is added one and one-half times its volume of dry ether. The substance is thus precipitated and, after washing in large volumes of alcohol-ether mixture containing alcohol and ether in the above mentioned proportions, may be dried over sulphuric acid at low temperatures and pulverized in a dry atmosphere.

The properties of tethelin may be summarized as follows: It is markedly hygroscopic, absorbing water rapidly from moist air. On standing after pulverization in contact with air in the presence of traces of moisture it darkens perceptibly in color and its iodine absorption value decreases. This decomposition is accelerated by warming. If packed in evacuated glass tubes, however, and perfectly dry, it may be heated to 80°C. without any perceptible discoloration.

Tethelin is soluble in water to the extent of about 5 per cent. It is also soluble in alcohol, ether, chloroform, and carbon tetrachloride. It is insoluble in a mixture of one part by volume of absolute alcohol and one and one-half parts by volume of dry ether. It contains 1.4 per cent of phosphorus, and nitrogen in the proportion of four atoms of nitrogen for every atom of phosphorus, two of the atoms of nitrogen being present in amino groups and one in an NH group which is converted into an amino group by hydrolysis with barium hydroxide. It yields unsaturated fatty acid soaps of barium on hydrolysis with barium hydroxide, and among the products yielded by hydrolysis with barium hydroxide followed by hydrolysis with dilute sulphuric acid is found *i*-inosite (hexahydroxybenzene).

Tethelin probably contains an imineazolyl group, and to this extent may be regarded as being related to the physiologically active substances of the posterior lobe of the pituitary body.<sup>2</sup> It is not, however, possessed of the characteristic physiological activity of these substances, relatively large doses administered intravenously to rabbits (50 mg. per kilo of body weight) producing only a very slight transient fall in blood pressure and no diuresis.

The action of tethelin upon the normal growth of mice is identical with the action of the whole anterior lobe.<sup>3</sup> It consists in a marked

<sup>2</sup> Barger, G., and Dale, H. H., *Jour. Physiol.*, 1910-11, xli, 499. Dale, H. H., and Laidlaw, P. P., *Jour. Physiol.*, 1911, xliii, 182. Aldrich, T. B., *Jour. Am. Chem. Soc.*, 1915, xxxvii, 203.

<sup>3</sup> Robertson, T. B., *Jour. Biol. Chem.*, 1916, xxiv, 385, 397.

retardation of the early (preadolescent) growth in weight (subsequent to 4 weeks after birth) and an equally marked acceleration of post-adolescent growth.

In a previous article<sup>4</sup> we have shown that the hypodermic administration of emulsified tissue of the anterior lobe of the pituitary body to rats, either directly into or in localities remote from the tumors, leads to a remarkable acceleration of the growth of the Flexner-Jobling carcinoma, especially during the period of growth between the 20th and 37th days succeeding inoculation. This effect is specific, since similar administrations of liver tissue, during the same period, far from causing any acceleration of the growth of the tumors, actually resulted in a slight but definite retardation of their growth.

In view of these results it appeared of importance to ascertain whether tethelin also reproduces the effect of the whole anterior lobe upon the growth of carcinomata, and to that end the investigations which are about to be described were undertaken. At the same time it seemed advisable to ascertain whether any other alcohol-soluble extractive of the anterior lobe of the pituitary body exerts any action upon the growth of carcinomata. Three such fractions were prepared and their action upon the growth of carcinomata was investigated.

*Fraction A.*—Tethelin was removed from the alcohol extract by precipitation by ether as described above, and the supernatant fluid was syphoned off from the precipitate and filtered. The ether with part of the alcohol was then distilled off at atmospheric pressure (without exposure to the air) until the boiling point of the mixture reached 78° C. The solution, much diminished in volume, was now cooled by standing in the ice chest for a couple of days. A small quantity of material, slightly soluble in cold alcohol and only partially soluble in ether, was deposited. This was washed in cold alcohol and dried over sulphuric acid. This substance, which behaved like lecithin in forming emulsions with water, composed Fraction A. It probably consisted in great part of a mixture of phospholipins.

*Fraction B.*—The solution obtained after the separation of Fraction A was evaporated to dryness on a water bath in a vessel provided only with a small exit for the alcohol vapor. The residue was taken up in a small quantity of ether, in which it was completely soluble, and several volumes of acetone were added to this solution. The precipitate was collected on a filter, washed with

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<sup>4</sup> Robertson, T. B., and Burnett, T. C., *Jour. Exper. Med.*, 1915, xxi, 280.

acetone and dried over sulphuric acid. This formed Fraction B; it consisted, probably entirely, of lecithins.

*Fraction C.*—The filtrate from the above was evaporated to dryness in a water bath in a vessel provided only with a small exit for the ether-acetone vapor. The residue, a dark colored substance, gummy when cool and soft and viscous when warmed, was dried over sulphuric acid. This formed Fraction C. It probably consisted largely of ordinary fats and also must have contained all the cholesterol and cholesterol esters originally present in the tissue. On triturating with water it formed an unstable emulsion.

From a number of successive batches of 300 anterior lobes, each weighing approximately 450 gm. (fresh tissue), were obtained the following approximate yields of the above described substances.

	gm.
Tethelin .....	2.6-3.0
Fraction A.....	0.3-0.8
Fraction B.....	4.0-6.0
Fraction C.....	4.0-6.0

#### *History of the Tumors Employed.*

We propagated the Flexner-Jobling carcinoma by inoculation into the axillary region through five generations. The percentage of takes was high, varying between 60 and 90 per cent. Half grown or adult animals were employed to propagate the tumors and also in the experiments enumerated below, with the exception of a small number of younger animals evenly distributed among all the different experimental groups. As stated in our previous communication referred to above, this tumor, non-metastasizing when supplied to us by Dr. Peyton Rous, yielded metastases in the first generation in this laboratory and has continued to do so in succeeding generations.

#### *Influence of Tethelin upon the Growth of Carcinomata.*

73 white rats were inoculated in the axillary region with peripheral portions of a large rapidly growing tumor of our 5th generation from Rous's 21st generation. After 21 days 49 of the animals (67 per cent) were found to have well developed tumors. These were divided into two batches. One, consisting of 24 animals was retained as controls, and the 25 individuals comprising the other received, on the 21st, 23rd, 25th, 28th, 30th, and 32nd days after inoculation, 0.6 cc. each of a 5 per cent solution of tethelin in  $\frac{M}{8}$  sodium chloride, to which had been added 0.4 per cent of tricresol. The tethelin was injected hypodermi-

cally on the side remote from the tumor. The dose employed (30 mg.) corresponded to three anterior lobes, estimating the tethelin content of each anterior lobe to be 10 mg. No ill effects were observed to follow the administrations.

The tumors were measured through the skin in two diameters at right angles to each other on the dates enumerated below (Table I).

The mean of the two diameters (usually the longest and shortest) was recorded as the average diameter of any given tumor, and the average of these estimates was regarded as the average diameter of the tumors in any given group of animals.

The average diameter of the tumors in the control animals on the 21st day after inoculation was 12.4 mm., that of the tumors in the animals reserved for treatment was 12.1 mm. Calling these initial diameters in each group 100, and referring each of the subsequent measurements to this unit of comparison, we obtained the following results.

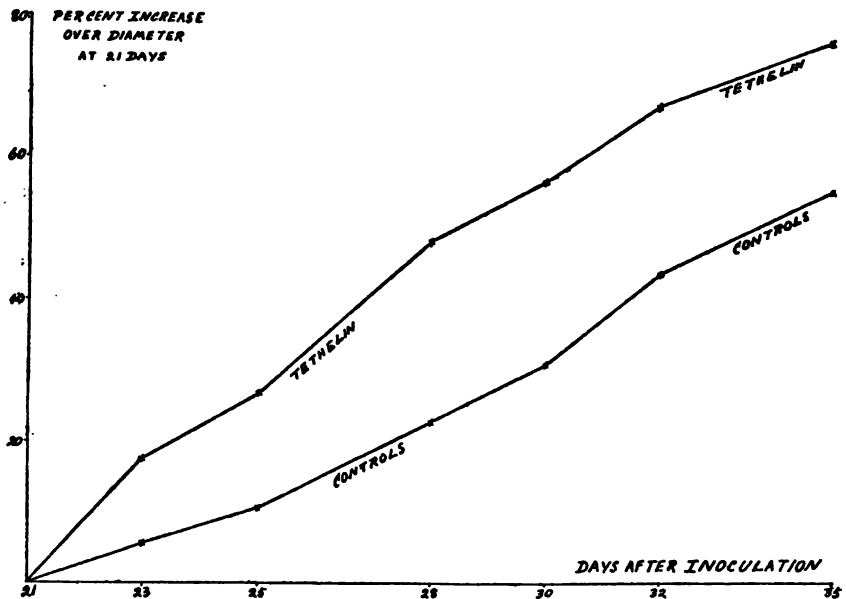
TABLE I.

Days after inoculation.	Diameter of tumors in controls.	Diameter of tumors in animals receiving tethelin.
21	100.0	100.0
23	105.6	117.4
25	110.5	126.4
28	122.6	147.9
30	130.6	156.2
32	143.5	167.0
35	154.9	176.0

These results are depicted graphically in Text-fig. 1. It will be seen that the administration of tethelin caused marked acceleration of the growth of the primary tumors, their linear growth between the first and fifth administration being from two to three times as rapid as that of the controls.

On the 63rd day all the surviving animals (23 of each group) were killed and the viscera examined for metastases. 2, or 9 per cent, of the control animals were found to have developed metastases, while 8, or 35 per cent, of the treated animals had developed metastases. The administration of tethelin, therefore, markedly enhances

the tendency of the tumor to form metastases, the differing result obtained by us with whole anterior lobe administration<sup>4</sup> being probably attributable to the relatively much smaller doses (only 0.5 gm., one-third of an anterior lobe in each dose) employed by us in our previous experiments.



TEXT-FIG. 1. The acceleration of the growth of carcinomata by hypodermic administrations of tethelin.

*The Influence of Other Alcohol-Soluble Extractives of the Anterior Lobe of the Pituitary Body upon the Growth of Carcinomata.*

140 white rats were inoculated in the axillary region with peripheral portions of a large tumor taken from one of the controls in the preceding experiments; *i.e.*, a tumor of our 6th generation from Rous's 21st generation. After 21 days 105 of the animals (75 per cent) were found to have well developed tumors. These were divided without selection into four batches. One, consisting of 27 animals, was retained as controls; the remaining three groups, consisting of 26 animals each, were treated with Fractions A, B, and C, respectively, on the 21st, 23rd, 25th, 28th, 30th, and 32nd days after inoculation. In each case the substance was administered hypodermically on the side of the animal remote from the tumor. Watery emulsions in  $\frac{M}{8}$  sodium chloride solution containing 0.4 per cent of tri-

cresol were employed. The emulsion of Fraction A contained 1.7 per cent of the substance, while the emulsions of Fractions B and C each contained 5 per cent of these substances. The dose administered was in each case 0.6 cc. of the emulsion. No ill effects were observed to follow any of the administrations.

The average diameter of the tumors in the control animals on the 21st day after inoculation was 13.1 mm. The diameters of the tumors in the animals subsequently treated with Fractions A, B, and C were 11.3, 13.4, and 11.1 mm., respectively. Calling these initial diameters in each group 100, and referring each of the subsequent measurements to this unit of comparison, we obtained the following results.

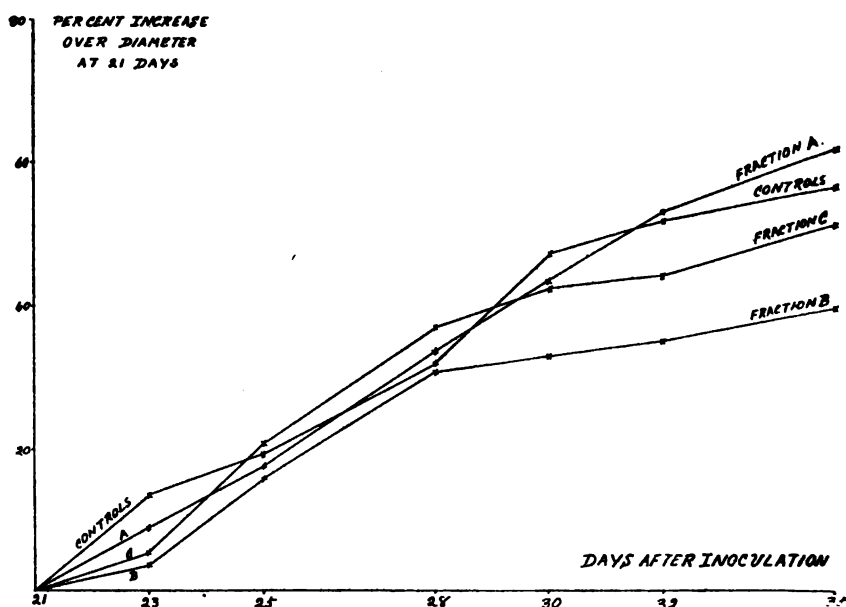
TABLE II.

Days after inoculation.	Diameter of tumors.			
	Controls.	Fraction A.	Fraction B.	Fraction C.
21	100.0	100.0	100.0	100.0
23	113.7	108.8	103.7	105.4
25	119.1	117.7	115.7	120.7
28	132.1	133.6	130.6	136.9
30	147.3	143.4	132.8	142.3
32	151.9	153.1	135.1	144.1
35	156.5	161.9	139.6	151.3

These results are depicted graphically in Text-fig. 2. It will be seen that neither Fraction A nor Fraction C exerted any significant action upon the growth of the tumors. Fraction B, however, caused a marked retardation of the growth of the primary tumors. It will be recollected that Fraction B, consisting as it does of substances soluble in alcohol and in ether, and precipitable by acetone, is composed almost entirely of lecithin. The retardation of tumor growth following its administration is therefore merely confirmatory of our previous finding<sup>5</sup> that administrations of lecithin markedly diminish the rate of growth of the Flexner-Jobling carcinoma in rats. The lecithin employed in our previous experiments, however, was prepared from yolks of eggs by extracting them with ether and adding acetone to this extract. It therefore probably consisted of a mixture

<sup>5</sup> Robertson, T. B., and Burnett, T. C., *Jour. Exper. Med.*, 1913, xvii, 344.

of approximately equal parts of lecithin and cephalin,<sup>6</sup> while the lecithin employed in the experiments just described, owing to the manner of its preparation (extraction of the tissue with alcohol) cannot have contained any admixture of cephalin. Evidently, therefore, lecithin itself is capable of causing the retardation observed in these and our previous experiments. Whether cephalin is capable of



TEXT-FIG. 2. The effect of hypodermic administrations of alcohol-soluble extracts from the anterior lobe of the pituitary body (other than tethelin) upon the growth of carcinomata. Note the retardation due to the lecithin fraction (Fraction B).

exerting a like retardation or whether it only acted in our previous experiments as an inert diluent of the lecithin remains to be ascertained at some future date.

In our previous experiments, moreover, the lecithin was administered by direct injection into the tumors. In the present experiments the lecithin was injected at a locality remote from the tumors.

<sup>6</sup> Stern, M., and Thierfelder, H., *Ztschr. f. physiol. Chem.*, 1907, liii, 370.



Evidently the retarding effect of lecithin is not attributable to a purely local action.

Since Fraction C did not exert an accelerative action upon the growth of the tumors, its content of free cholesterol was presumably small;<sup>6</sup> no analytical determinations have, however, been made.

On the 49th day the surviving animals (twenty-five of each group) were killed and the viscera examined for metastases. The control group and the groups treated with Fractions A and B, respectively, each yielded three animals, or 12 per cent, which had developed metastases. The group treated with Fraction C yielded five animals with metastases, or 20 per cent. It is possible that this slightly enhanced tendency to metastasize may have been attributable to the cholesterol content of this fraction.

#### CONCLUSIONS.

1. The hypodermic administration of tethelin increases markedly the rate of growth of the primary tumor and the tendency to form metastases in rats inoculated with carcinoma, in this, as in other respects, reproducing the action of the whole anterior lobe of the pituitary body.

2. Other alcohol-soluble extractives of the anterior lobe of the pituitary body, with the exception of the lecithin fraction, exert no appreciable effect upon the growth of carcinomata in rats.

3. The lecithin fraction, as in previously reported experiments in which we employed lecithin obtained from eggs, causes evident retardation of the growth of carcinomata in rats.

In conclusion we desire to express our indebtedness to Mr. Ralston B. Brown, Superintendent of the Oakland Meat and Packing Company, to whose cooperation we owe the supply of pituitary glands which has afforded us the opportunity of carrying out these investigations.



# THE INTRAVENOUS INJECTION OF MAGNESIUM SULPHATE FOR ANESTHESIA IN ANIMALS.

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(Received for publication, March 10, 1916.)

The effect on animals of intravenous injections of magnesium sulphate was investigated by us from a general experimental point of view about ten years ago.<sup>1</sup> The use of this salt for practical purposes of anesthesia in human beings was first studied by Haubold and Meltzer by the intraspinal method.<sup>2</sup> About two years ago a combination of subminimal doses of magnesium sulphate intramuscularly and ether by intratracheal insufflation was found by the present writers<sup>3</sup> to be effective in animals, and by Peck and Meltzer and also Elsberg and Meltzer in human beings. The use of magnesium sulphate by intravenous injection was in general discouraged by one of us.<sup>4</sup> However, a series of experiments made by the present writers with intravenous injection of magnesium sulphate in cases of experimental tetanus,<sup>5</sup> and the meager but satisfactory experience which Kohn<sup>6</sup> and Straub<sup>7</sup> had with the employment of this method in cases of tetanus in human beings, induced us to take up the experimental study in animals of the employment of magnesium sulphate by intravenous injection for the purpose of producing anesthesia. This was done as a preliminary test for the admissibility of

<sup>1</sup> Meltzer, S. J., and Auer, J., *Am. Jour. Physiol.*, 1905-06, xv, 387.

<sup>2</sup> Haubold, H. A., and Meltzer, S. J., *Jour. Am. Med. Assn.*, 1906, xlv, 647.

<sup>3</sup> Meltzer, S. J., and Auer, J., *Proc. Soc. Exper. Biol. and Med.*, 1912-13, x, 159; *Zentralbl. f. Physiol.*, 1913-14, xxvii, 632.

<sup>4</sup> Meltzer, S. J., *Berl. klin. Wchnschr.*, 1915, lii, 261.

<sup>5</sup> To be published later.

<sup>6</sup> Kohn, H., *Berl. klin. Wchnschr.*, 1915, lii, 89.

<sup>7</sup> Straub, W., *München. med. Wchnschr.*, 1915, lxii, 341.

studying the exclusive use of intravenous injections of magnesium sulphate as a means of producing, or at least inducing, anesthesia in human beings. We wish to record a few abbreviated protocols of these experiments.

#### EXPERIMENTAL.

The experiments were made on dogs. The left external jugular vein was exposed under local anesthesia by ethyl chloride, a cannula introduced, and magnesium sulphate in an  $\frac{M}{4}$  solution injected through the cannula from a burette. The reaction of the animals was tested in various ways, as indicated in the protocols. When the respiration appeared to be shallow, pharyngeal insufflation<sup>a</sup> was employed either temporarily or throughout the entire experiment. In some instances intratracheal insufflation was given, especially for the purpose of testing the possibility of introducing the intratracheal tube without using any other anesthetic and while the animal was still breathing spontaneously. At the end of the experiment either a small quantity of a calcium chloride or sodium sulphate solution was injected, or no further injection was given.

*Experiment 1.*—Black and white female fox-terrier; weight 4,600 gm.

10.20. On electric warming pad at medium. Clip hair of neck; used ethyl chloride as local anesthetic for exposing and inserting cannula in external jugular vein.

10.45. Rectal temperature 38.8°C.

10.50. Start infusion of  $\frac{M}{4}$  magnesium sulphate into jugular vein.

10.55. Operation completed.

11.00. 5 cc. Lid reflex prompt and strong.

11.04. 14 cc. Slightly restless.

11.05½. Lid reflex prompt; active expiration, of good strength.

11.08. 20.5 cc. Lid reflex prompt and strong. Respiration good, fairly rapid, active expiration.

11.11. 26 cc. Respiration fairly rapid; quiet; lid reflex prompt and strong.

11.13. 33 cc. Respiration slower, with active expiration; no sound. Heart slow.

11.15. Catheter F. 19 inserted into trachea with ease; start air insufflation.

11.17. 40.5 cc. Lid reflex slight.

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<sup>a</sup> Meltzer, S. J., *Jour. Am. Med. Assn.*, 1913, lx, 1407; *Berl. klin. Wchnschr.*, 1915, lii, 425.

- 11.18. Very slight inspiration, slow rate; able to move head slightly.  
11.20. 44.5 cc. Very shallow respiration on stopping insufflation.  
11.22. 46.5 cc. Lid reflex fair.  
11.23. Occasionally spontaneous, fair expirations during insufflation.  
11.24. 50 cc. injected. Stop. Very slight spontaneous shallow respiration; slow rate. Lid reflex slight.  
11.25. Injected through venous cannula 15 cc.  $\frac{M}{4}$  calcium chloride into jugular vein. Respiration began promptly. Injection lasted about 1 minute. Suture wound after wiping with diluted tincture of iodine. Remove catheter. Animal trots away at once, slightly unsteady at first; wags tail, obeys command.  
12.20. Runs about with ease.  
2 days later, 10 a.m. Dog lively and active, jumps about, barks, behaves like a normal dog.  
Next day. Good condition.

The intravenous injection of an  $\frac{M}{4}$  solution of magnesium sulphate was given at a slow rate. After injecting 33 cc. in 23 minutes the intubation for intratracheal insufflation was performed with ease, while usually a great deal of ether has to be given to accomplish this purpose. A total of 50 cc. of magnesium was given in 34 minutes. The spontaneous respiration was then very shallow. After injecting 15 cc. of  $\frac{M}{4}$  calcium chloride the respiration recovered promptly and the animal would execute satisfactory voluntary movements.

*Experiment 2.*—Black and white mongrel, male; weight 5,850 gm.

Preliminary preparations as in Experiment 1.

- 11.37. Start infusion of  $\frac{M}{4}$  magnesium sulphate into jugular vein.  
11.41. 9 cc.  
11.44. 18 cc. Moves head, lid reflex strong. Barks sleepily.  
11.46. 22 cc. Respiration more rapid, shallower; no reaction to pricking skin with needle; lid reflex strong.  
11.50. 36 cc. Fair, slow respiration with active expiration. Lid reflex prompt. Occasional slight general motions. Heart slowed.  
11.52. 39.5 cc. Respiration slow, moves head.  
11.55. 46 cc. Very slow respiration, lid reflex fair; heart slow.  
11.57. 50 cc. Respiration very slight; start pharyngeal insufflation. Pulse improved.  
12.02. 54.5 cc. Stop insufflation to change stomach tube; no respiration seen.  
12.04. 55.5 cc. Heart 24 to  $\frac{1}{4}$  minute, small, regular, soft.  
12.06. 56.5 cc. Breathes spontaneously; no reaction to pricking skin with needle.

12.09. 61 cc. Tracheal catheter inserted; slow spontaneous respiration, fair depth, some active expiration; lid reflex slight, moves head.

12.12. 64 cc. Legs relaxed; spontaneous respiration fair; lid reflex slight.

12.15. Pulse 27 to  $\frac{1}{4}$ , regular, strong (femoral); fair spontaneous respiration. Stop insufflation. 65.5 cc. injected. Respiration gradually improves and becomes good, deep. Respiration moderately slow; start air again.

12.16. Excellent respiration; no lid or corneal reflex. Tongue pink.

12.20. Respiration easy, more rapid; no lid or corneal reflex. 70 cc. injected.

12.22. Moves head; increase magnesium flow slightly.

12.23. 73 cc. Respiration slower, but good depth. Femoral pulse 22 to  $\frac{1}{4}$ , respiration good depth.

12.27. No reaction to pricking skin. 78.5 cc. Stop magnesium.

12.28. 8 cc. of  $\frac{M}{8}$  calcium chloride into jugular vein. Respiration greatly improved and more rapid. Wound sutured. Placed on floor, holds head up; front legs spread, do not support body; licks jaws; moves tail on pressure. Pays attention to call and wags tail.

12.35. Able to walk about; tail erect.

2.10. Walks about normally.

Next day. Good condition.

The first 50 cc. injected in 20 minutes, about 2.5 cc. per minute, nearly completely abolished respiration. Pharyngeal insufflation was then started and exerted immediately a good effect. From 12.02 to 12.06 only 2 cc. were injected, equal to 0.5 cc. per minute; spontaneous respiration returned, but no reaction to pricking, and intubation of catheter was easily executed. Thereafter the rate of inflow was kept fairly low. Spontaneous respiration was continually present but was generally slow. There was no lid reflex and no reaction to pricking. After injecting about 78 cc. of magnesium in 50 minutes a quantity of 8 cc. of  $\frac{M}{8}$  calcium chloride was injected; respiration improved at once. Voluntary movements, however, returned gradually.

*Experiment 3.*—Male; weight 7,500 gm.

Was fed previous to experiment. Preparation as in previous experiments.

2.27. Rectal temperature 38.7°. Femoral pulse 33 to  $\frac{1}{4}$ , regular.

2.33. Start  $\frac{M}{4}$  magnesium sulphate into jugular vein.

2.37. 7 cc. Respiration deeper; swallows occasionally.

2.40. Pulse softer, 38 to  $\frac{1}{4}$ , regular; respiration more rapid with strong active expiration. 14 cc.

2.41. Vomited yellowish brown fluid.

2.43. Vomited large amount of yellow fluid with masses of meat.

2.44. 22 cc. Lid reflex prompt; occasional moderate struggle.

- 2.48. 32 cc. Moderate barks. Lid reflex strong.
- 2.49. 38.5 cc. Respiration chiefly expiratory. Tracheal catheter inserted; animal shows resistance. Catheter F. 21; constant air stream with remission. Lid reflex strong.
- 2.54. 44 cc. No reaction to pricking skin with needle.
- 2.55. 45 cc. Stop insufflation; practically only one inspiration and a number of weak abdominal contractions appeared during the intermission. Lid reflex strong. Able to move head moderately. Occasionally a sharp expiratory movement of abdomen. Started intratracheal insufflation again.
- 3.06. Remove tracheal catheter; start pharyngeal insufflation, tube in stomach.
- 3.10. 57 cc. Moves head vigorously; femoral pulse regular, soft.
- 3.13. 59.5 cc. Legs limp. Lid reflex good.
- 3.22. 73 cc. Open abdomen, rub peritoneum above liver; no motions.
- 3.23. 76 cc. Lid reflex fair.
- 3.26. 80 cc. Stop insufflation; no definite respiration, some expiratory contractions. Start insufflation. Abdomen closed.
- 3.31. 80 cc. No definite respiration on stopping insufflation. Good lid reflex. Pupils wide.
- 3.35. 90 cc. Stop magnesium. Lid reflex fair. Stop air; 3 slight respirations; start insufflation.
- 3.38. Slight, slow, shallow respiration on stopping pharyngeal insufflation. Femoral pulse 18 to  $\frac{1}{2}$ , regular, fair tension.
- 3.40. 10 cc. of  $\frac{M}{8}$  calcium chloride into jugular vein. Deep, slow respirations begin.
- 3.43. Pulse 29 to  $\frac{1}{2}$ , regular, strong tension; no reaction to pricking skin with needle. Lid reflex strong; pupils wide. Neck wound closed. Placed on floor, attempts to get up.
- 3.44. Gets up after a few trials.
- 3.55. Lies on side; no response to pressure on toes; wags tail; tolerates probe in nose for a short time. When placed on feet walks away fairly steadily, then lies down again.
- 4.25. As before; no response to pressure on legs; lies on side usually. Raises head on call; walks when placed on feet. Lid reflex prompt; drinks some water. Killed later by chloroform.

This animal was fed about 3 hours before the experiment was started. After 14 cc. of the magnesium solution were injected (in 7 minutes) the animal vomited. This indicates the central action of magnesium sulphate. After 45 cc. were injected (in 22 minutes) there was practically no spontaneous respiration, although the lid reflex was strong and the animal was able to move its head. The failure of the respiration in this case was undoubtedly due to the in-

hibitory action of the magnesium sulphate upon the respiratory center, and not to a paralysis of the motor nerve endings of the respiratory muscles, which, as a rule, remain excitable longer than the other skeletal muscles. After 80 cc. of the magnesium solution (in 58 minutes) the abdomen was opened and the sensitive parts of the parietal peritoneum were rubbed without eliciting any reaction, although the lid reflex was still good. This animal received 90 cc. of the magnesium solution in 62 minutes. 3 minutes after stopping the injection the animal had only slow and shallow respirations. The injection of 10 cc. of  $\frac{M}{8}$  calcium chloride deepened the respirations; it exerted also, fairly promptly, a favorable effect upon the general motility of the animal. But the return of reactions to a probe inserted into the nose and to other sensory stimuli was slow.

*Experiment 4.*—Wolf hound, female; weight 6,700 gm.

Preliminary preparations same as in Experiment 1.

2.42. Start  $\frac{M}{4}$  magnesium sulphate into jugular vein.

2.43. 3 cc.

2.46. 10.5 cc. Lid reflex prompt, sustained. Respiration faster; pulse fuller, faster, 33 to  $\frac{1}{2}$ . Respiratory irregularity of pulse rhythm practically gone.

2.48. 16.5 cc. Respiration 18 to  $\frac{1}{2}$ , fair depth, slight active expiration.

2.50. 22 cc. Lid reflex prompt, but no longer sustained closure.

2.52. 28 cc. Respiration good, 14 to  $\frac{1}{2}$ ; femoral pulse 30 to  $\frac{1}{2}$ , good volume and tension; no irregularity.

2.55. 36 cc. Respiration slower; pain sensation of skin abolished.

2.56. 39 cc. Respiration slower, but good depth; pulse irregular. Start pharyngeal insufflation. Stomach tube.

2.58. 46 cc. Lid reflex prompt but slight. Pupils wide.

3.00. 47 cc. Open abdomen and rub diaphragm; no motion of any kind.

3.02. Stop insufflation; very slight shallow respiration; start air; lid reflex a slight flick. Pulse 36 to  $\frac{1}{2}$ , regular, small volume and tension. Rub peritoneum; no sign of movement; legs limp.

3.07. 51 cc. Lid reflex slightly better; rubbing peritoneum causes no motion of any kind. No knee jerk.

3.08 $\frac{1}{2}$  52.5 cc. Stop insufflation; 16 very slight respirations per  $\frac{1}{2}$ . Rub diaphragm and parietal peritoneum: no motion. Legs limp. Lid reflex fairly sustained now (closure).

3.15. 56.5 cc. Femoral pulse 32 to  $\frac{1}{2}$ , regular, small, and soft.

3.17. 57 cc. Stop air; no respiration in 35 seconds. Start air. Lid reflex slight; pupils moderately contracted.

3.21. 58.5 cc. Rub peritoneum and diaphragm; slight motion of leg, but no other perceptible movement. Spontaneous respiration noticeable during insuffla-



tion. Stop air; 24 shallow respirations per  $\frac{1}{2}$ . Start air. Pulse 32 to  $\frac{1}{2}$ , fair volume and better tension now. Lid reflex prompt but weak.

3.25. 59.5 cc.

3.26. Moves leg slightly.

3.29. 61 cc. On rubbing peritoneum and diaphragm no motion, but later made vigorous movements with head and leg. (No apparent relation to stimulus; rubbing repeated; no motion.)

3.31. Some strong movements of head and legs. Lid reflex flick, not sustained. Stop air. Respiration spontaneous, 15 to  $\frac{1}{2}$ , good depth. Stop insufflation entirely.

3.33. 63 cc. of magnesium. Stop. Excellent respiration. Suture abdominal wound. Ligate jugular vein and suture wound in neck. No calcium chloride given.

3.36. Placed on floor; attempts to walk; hind legs spread; raises head and looks about.

3.43. Lying on side; placed on legs; walks about; lies down again shortly. Withdraws legs fairly promptly when pressed; walks away when tail is pressed; no sign of pain.

4.00. Walks about. Rectal temperature 36.4°; pulls away leg when toes are pressed.

4.38. Killed by chloroform.

During the first period of the experiment (about 18 minutes) the inflow of the magnesium solution occurred at a rate of about 2.9 cc. per minute. After the injection of 36 cc. (in 13 minutes) skin sensibility was abolished; the respiration, though slower, was good and there was even an occasional struggle. The pharyngeal insufflation was started before there was any necessity for it. After the injection of 47 cc. (in 18 minutes) the abdomen was opened and the peritoneum rubbed without any reaction. About this time, however, the spontaneous respiration was shallow, lid reflex slight, and the legs were limp. In the following half hour the rate of injection was considerably reduced—about 16 cc. in 33 minutes. The loss of sensibility lasted for about half an hour longer. The spontaneous respiration returned perceptibly sooner. Altogether 63 cc. of magnesium sulphate were injected in about 51 minutes. Then, when the magnesium sulphate injection was stopped and the insufflation discontinued, the spontaneous respiration immediately appeared to be excellent. No calcium chloride was given. After placing the animal on the floor, motility and sensibility returned fairly soon. The quan-

tity of magnesium given in this experiment was not large and the rate of injection slowed down considerably during the latter part of the experiment. The recovery here was prompt and without the aid of calcium chloride.

*Experiment 5.*—Bull terrier, female; weight 7,600 gm.

Preparation the same as in previous experiments.

Rectal temperature 40.2°. Femoral pulse 30 to  $\frac{1}{2}$ , small, regular, good tension. Respiration slow, 12 to  $\frac{1}{2}$ , with active expiration.

10.58. Start  $\frac{M}{4}$  magnesium sulphate into jugular vein.

11.04. 17 cc. Lid reflex prompt but not sustained; pupils wide. Respiration less deep, slow. Swallows occasionally.

11.06. 25 cc. 14 respirations to  $\frac{1}{2}$ , good depth, moderate active expiration.

11.07. 29 cc. Lid reflex prompt and sustained. Quiet.

11.08. 35 cc. Pulse small, fairly soft, 38 to  $\frac{1}{2}$ , regular. Respiration good depth.

11.10. 43 cc. Respiration slow, less deep but still good, 7 to  $\frac{1}{2}$ . Slow magnesium inflow.

11.13. 50 cc. Respiration improved.

11.15. 53 cc. Magnesium inflow slowed. Lid reflex fairly prompt.

11.18. 56 cc. Open abdomen; rub peritoneum and diaphragm; no motion of any kind. Respiration good, more rapid than before.

11.20. 58.5 cc. Lid reflex slight; respiration rapid, 36 to  $\frac{1}{2}$ , next count 28 to  $\frac{1}{2}$ ; legs relaxed. Expose left sciatic nerve; no motion at first, later moderate general movements.

11.25. 71 cc. Respiration excellent.

11.29. 86 cc. Respiration much shallower and slower. Start pharyngeal insufflation.

11.32. 98 cc. No lid reflex.

11.34. 102 cc. No definite respiration.

11.37. 104.5 cc. Stimulated left intact sciatic with Petzold inductorium. At coil distances of 200 and 120 mm. no reaction elicited. At 80 mm. respirations appeared during stimulation; left toes moved slightly; also weak general motions and movements of tail. Rub peritoneum and diaphragm; no response.

11.42. 107 cc. Stop pharyngeal insufflation; slight spontaneous respiration present; start pharyngeal insufflation; increase magnesium flow slightly. Limp. No lid reflex; pupils very wide.

11.47. Abdomen and thigh wound sutured. 116 cc. No lid reflex.

11.49. 117 cc. Stop magnesium. Spontaneous respiration very slight; pharyngeal insufflation necessary.

11.50. 60 cc.  $\frac{M}{4}$  sodium sulphate into jugular vein. Respiration improved promptly. Stop pharyngeal insufflation.

11.53. Pulse good. Suture neck wound, lid reflex slight.

11.56. Placed on floor, holds head up for short time, then rests it on floor; cannot stand. Wags tail when called.

12.01. Pressure on tail and toes; moves head towards tail, draws away foot.

12.05. Lid reflex very slight, pupils wide. Able to get up but prefers to squat or lie down.

1.55. Walks about readily, keeping left hind leg lifted (left sciatic nerve had been exposed), no staggering; lid reflex prompt and sustained. Pupils well contracted. Urinated large amount; first time since injection.

4.45. Walks about easily when placed on feet (staid in one place since last note); no more urine passed. Killed by chloroform.

This dog had from the start a slow respiration although its temperature was higher than normal. During the first 10 minutes of the magnesium infusion 3.5 cc. per minute were injected, more than in any of the animals in previous experiments. There were no struggles. After injecting 56 cc. the abdomen was opened and the parietal peritoneum rubbed without any reaction, while the respiration was good and even more rapid than before. After 102 cc. no definite respirations were present, and after 104 cc. the motor nerve endings were affected. The inflow was then reduced—only 13 cc. in 12 minutes. Altogether 117 cc. were injected in 51 minutes. There was practically no spontaneous respiration when the infusion of the magnesium solution was discontinued. However, the respiration improved within 1 minute after the injection of 60 cc. of  $\frac{M}{4}$  sodium sulphate. The general motor and sensory depression seemed also favorably affected by this injection.

*Experiment 6.*—Black male; weight 7,500 gm.

Preliminary preparation as in Experiment 1.

3.13. Start  $\frac{M}{4}$  magnesium sulphate into jugular vein.

3.17. 12 cc. Respiration 8 to  $\frac{1}{4}$ . Good depth. Femoral pulse 29 to  $\frac{1}{4}$ , regular, good volume and tension.

3.18. 15 cc. Lid reflex prompt and strong, pupil moderately dilated. Dog quiet.

3.20. 25 cc. Good respiration, 12 to  $\frac{1}{4}$ , active expiration stronger. Femoral pulse 32 to  $\frac{1}{4}$ , regular, good tension. Barks.

3.22. 31 cc. Lid reflex prompt and sustained; pupils wider.

3.23. 34.5 cc. Pain abolished; opening of peritoneum; respiration easy, good depth and frequency. Abdomen relaxed.

3.25. 46 cc. Rub peritoneum and diaphragm; no movement. Lid reflex prompt and sustained; respiration slow, good depth, 11 to  $\frac{1}{4}$ .

3.27. 50 cc. Blood bright red. Slow magnesium inflow.

- 3.28. 51 cc. Rub peritoneum of diaphragm; no movement. Lid reflex weak.
- 3.30. Pulse 30 to  $\frac{1}{2}$ , regular, good volume and tension.
- 3.31. 53.5 cc. Slight knee jerk.
- 3.32. 55 cc. Moved legs; respiration faster and deeper. Rub peritoneum and diaphragm; no immediate effect, after a few seconds rapid respiration with moderate strength. Increase magnesium inflow.
- 3.34. 61 cc. Lid reflex a mere flick; rapid respiration with active expirations in short group, then easy respirations without active expirations.
- 3.39. Respiration slow, good depth with active expiration.
- 3.42. 85 cc. Blood a little darker; respiration shallower.
- 3.44. Start pharyngeal insufflation.
- 3.46. Pulse 20 to  $\frac{1}{2}$ , fair volume and tension, regular. No lid reflex. Rub peritoneum and diaphragm; no movement. Legs limp; no knee jerk.
- 3.52. No spontaneous respiration on stopping insufflation.
- 3.55. Pupils well dilated but not maximal; no lid reflex. 103 cc. Pulse small, 25 to  $\frac{1}{2}$ .
- 4.00. 104.5 cc. No lid reflex. Rub peritoneum; no movement; no knee jerk.
- 4.05. No spontaneous respiration on stopping insufflation; pulse weak. Start insufflation again.
- 4.07. No movement on rubbing peritoneum and diaphragm. 106 cc. Stop magnesium. No lid reflex. Injected 60 cc.  $\frac{M}{4}$  sodium sulphate into jugular vein.
- 4.10. Femoral pulse 25 to  $\frac{1}{2}$ , small, regular, better tension. Rubbing peritoneum and diaphragm; no movement.
- 4.11. Stop insufflation; slow respiration, getting deeper; start insufflation again. No lid reflex; pupil wide.
- 4.13. Rub peritoneum; no movement. Legs limp; no knee jerk.
- 4.17. Suture abdomen and neck wound and stop insufflation. Pulse 27 to  $\frac{1}{2}$ , regular, good volume and tension; 4 respirations to  $\frac{1}{2}$ , good and deep, no active expiration; no lid reflex, pupils wide; 36.4°. Placed on floor; cannot stand, lies on side. Pain sensation fair; looks about and wags tail.
- 4.25. No lid or corneal reflex; wags tail when called; feeble knee jerk; on moderate pressure of toe pads, no movement. When lifted and placed on floor front legs bear body weight, but not the hind legs. Pupils widely dilated.
- 4.30. Respiration easy, good depth, 14 to  $\frac{1}{2}$ , no active expiration. Femoral pulse 29 to  $\frac{1}{2}$ , regular, good volume and tension. Lies on side, wags tail. Very slight lid reflex.
- 4.45. Sits up on haunches, but does not walk about; lid reflex fairly good. Passed small amount of urine.
- 5.00. Walks about, no weakness; lid reflex prompt and sustained; pupils still wide. Killed with chloroform.

This strong dog received in the first 10 minutes about 35 cc. of the magnesium solution. There was very little excitation, and at the

end of this period the skin of the abdomen could be incised to the peritoneum without any reaction. In the following 2 minutes 11 cc. were infused, and the sensitive parts of the parietal peritoneum were energetically rubbed without producing any reaction, while respiration was still good and the lid reflex prompt and sustained. The rate of injection was now reduced, and the spontaneous respiration kept up efficiently for some time. After 19 minutes during which time about 44 cc. were injected (a little less than 2.5 cc. per minute) pharyngeal insufflation was started. In the next 23 minutes only about 16 cc. were injected (about 0.7 cc. per minute). During this period there were no spontaneous respiration, no lid reflex, no knee jerk, and the legs were limp; finally the pulse became weaker. Altogether 106 cc. of the magnesium solution were injected in 54 minutes. At the end of the magnesium injection no calcium chloride was given, but, as in the previous experiment, 60 cc. of sodium sulphate in  $\frac{1}{4}$  solution were injected intravenously. The effect of the injection in this experiment, however, was in no way striking. The respiration did not improve at once and the insufflation had to be continued for about 10 minutes longer. The fact should be borne in mind that in this experiment the rate of injection of the magnesium sulphate during the first half of the infusion period was considerably greater than in any of the other experiments.

In addition to the foregoing experiments we wish to record briefly the exceptional course of one of the experiments. This dog had an irregular heart beat and its extremities were rigid before the experiment was begun. There was no spontaneous respiration after injecting 44 cc. of magnesium solution (in 14 minutes), while the peritoneum remained sensitive and the lid reflex active during most of the injection period. The animal received 72 cc. in 57 minutes. The pulse was small and often weak during the last half hour. At the end of the magnesium injection 10 cc. of  $\frac{1}{8}$  calcium chloride were injected without restoring the spontaneous respirations. A few minutes later 5 cc. more of the calcium chloride brought on some weak respirations, but the heart stopped soon after and the animal died.

Here was a case in which calcium did not restore the respiration which had been abolished by magnesium; on the contrary, it was perhaps instrumental in accelerating cardiac death.

## SUMMARY AND CONCLUSIONS.

These experiments justify the following general conclusions.

By the intravenous injection of  $\frac{N}{4}$  magnesium sulphate into dogs at a certain rate, a stage can be reached where the abdominal walls are completely relaxed and when section of the abdomen and stimulation of sensitive parts of the parietal peritoneum do not produce pain or elicit any reaction of the animal. At the same time spontaneous respiration may still be maintained within normal limits and the lid reflex be fair or even normal. In this stage intratracheal intubation for artificial respiration can be easily accomplished. This stage may be attained in 12 to 14 minutes when the rate of injection is about 3 cc. per minute. When this stage is once attained the rate of injection should gradually be reduced, otherwise, sooner or later, spontaneous respiration will be abolished, and by a further maintenance of the rate of injection all the skeletal muscles may become paralyzed.

When the injection of magnesium is continued for a longer period, the paralytic effects of the magnesium injection will set in, even when administered at a slow rate.

The paralysis of the respiratory function is readily met by intrapharyngeal insufflation, which is easily executed even without training in this procedure, or by the method of intratracheal insufflation, if executed by one trained in its management.

When the respiration of the animal is accomplished by insufflation, the paralytic effect of the magnesium may be abolished fairly rapidly by an intravenous injection of about 10 cc. of an  $\frac{N}{8}$  calcium chloride solution; or it may disappear slowly, after the infusion of the magnesium solution is discontinued for some time. The latter mode of disappearance may be favorably accelerated by an intravenous infusion of 60 to 100 cc. of an  $\frac{N}{4}$  solution of sodium sulphate.

The production of anesthesia by intravenous injection of magnesium sulphate should not be undertaken unless an apparatus for intrapharyngeal insufflation is at hand, because in exceptional cases the disappearance of spontaneous respiration may be one of the earliest consequences of the magnesium injection.

The injection of calcium chloride should not be employed in cases in which the subject shows cardiac insufficiency. In such instances, moreover, injections of magnesium should not be used for the purpose of anesthesia; at least not until greater experience has been acquired in the employment of this method.





AN EXPERIMENTAL STUDY OF THE ADDITIVE AND  
ANTAGONISTIC ACTIONS OF SODIUM OXA-  
LATE, AND SALTS OF MAGNESIUM AND  
CALCIUM IN THE RABBIT.

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PLATE 95.

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INTRODUCTION

On the basis of the hypothesis that magnesium favors inhibition of the various functions of the nervous system, Meltzer and Auer studied extensively in this laboratory the action of magnesium salts upon various animals. In injecting magnesium sulphate subcutaneously,<sup>1</sup> they found that a certain dose, which varies with the species of animals, is capable of producing profound anesthesia and paralysis from which the animal recovers. For rabbits this dose amounts to about 1.5 gm. of magnesium sulphate ( $\text{MgSO}_4 + 7 \text{H}_2\text{O}$ ) administered in a molecular solution. Larger doses cause the death of the animal, as a rule, by respiratory paralysis. With an effective but non-fatal dose in subcutaneous injections the development of the depressing, inhibitory effect is gradual and fairly slow. When the maximum is reached, the turn for the recovery sets in soon; there is practically no real plateau to the inhibitory curve. The descending limb of this curve—the recovery—is steeper than the ascending one. When a magnesium salt is injected intramuscularly, the inhibitory as well as the fatal effects set in more promptly and with smaller doses.

In the course of their studies, Meltzer and Auer<sup>2</sup> found that calcium, which is chemically closely related to magnesium, is biologically appar-

<sup>1</sup> Meltzer, S. J., and Auer, J., *Am. Jour. Physiol.*, 1905, xiv, 366.

<sup>2</sup> Meltzer, S. J., and Auer, J., *Am. Jour. Physiol.*, 1908, xxi, 400.

ently the antagonist of the latter. When calcium is injected intravenously, shortly before or immediately after the respiration stops, into an animal which has received a fatal dose of magnesium, the animal will recover in less than a minute, provided, of course, that the circulation is still effective during the calcium injection. This biological antagonism is a remarkable fact and can be made the basis of many problems worth investigating. So far, at least, it is not known that between calcium and magnesium salts which have the same anion a chemical antagonism exists; no precipitation, for instance, occurs *in vitro* when a solution of magnesium chloride is mixed with a solution of calcium chloride. Calcium chloride is nevertheless strikingly antagonistic to magnesium chloride as far as the life of animals and plants is concerned.

What effect would the deprivation of the animal body of some of its calcium have upon the behavior of the animal? There are a number of acids and salts which precipitate calcium compounds *in vitro*. Will the administration of these calcium-precipitating compounds, let us say oxalic acid or oxalates in general, bring out symptoms indicating an increase of magnesium action? By precipitating calcium within the body a certain amount of unantagonized magnesium would be set free. Would this fact become manifest by the appearance of inhibitory and paralytic phenomena? The symptoms of oxalate poisoning do not speak for it; in general they possess rather the opposite character: excitation, tremor, and convulsions. But the amount of magnesium thus set free and the inhibition which it may be capable of exerting, might under these circumstances be too small to play a perceptible part, in the presence of the violent opposite symptoms which are brought out by another exciting factor of the oxalate. Could, however, the depressing component of the calcium-precipitating oxalate be brought out by a simultaneous administration of a subminimal dose of a magnesium salt? This was the problem which we tried to solve experimentally.

While we were at work on this problem, Schütz<sup>3</sup> published a brief preliminary communication in which he says that the susceptibility to magnesium injections could be increased occasionally, but not

<sup>3</sup> Schütz, J., *Wien. klin. Wchnschr.*, 1913, xxvi, 745.

constantly by sodium oxalate. A few months later Starkenstein,<sup>4</sup> with whose work we were not familiar until after we had given a preliminary communication of our results,<sup>5</sup> stated in a preliminary report that he found "like Schütz that the addition of oxalates constantly gave a visible increase of the magnesium narcosis."

We shall describe briefly our experiments bearing upon the problem under discussion and the conclusions to which they point.

#### EXPERIMENTAL PART.

We experimented exclusively on rabbits. Magnesium sulphate in M solution ( $\text{MgSO}_4 + 7 \text{H}_2\text{O}$ ) and sodium oxalate in 3 per cent solution<sup>6</sup> were injected separately and practically simultaneously, either into the lumbar muscles on opposite sides of the spine, or subcutaneously into each flank, the injection being usually followed by brief massage. All doses were estimated and are here reported in gm. of the salt per kilo of body weight. Most of the experiments were performed on a series of three animals, two serving as controls and receiving subtoxic doses of either magnesium sulphate or sodium oxalate alone. The experimental animal received the same dose of both salts.

#### *Intramuscular Injections.*

An abbreviated typical protocol follows.

#### *Experiment I.*

*Rabbit A.*—Oct. 2, 1913. Magnesium sulphate alone. Grey female. Weight 1,550 gm.

11.10 Right lumbar muscles: magnesium sulphate M, 4.3 cc. = 0.7 gm. per kilo of body weight.

11.23. Lying down, head up, breathing rapidly.

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<sup>4</sup> Starkenstein, E., *Wien. klin. Wchnschr.*, 1913, xxvi, 1235.

<sup>5</sup> Gates, F. L., and Meltzer, S. J., *Zentralbl. f. Physiol.*, 1913-14, xxvii, 1169. Starkenstein, E., *Zentralbl. f. Physiol.*, 1914, xxviii, 63; *Arch. f. exper. Path. u. Pharm.*, 1914, lxxvii, 45.

<sup>6</sup> Merck's reagent, "Sørensen's oxalate." Impure oxalates are not soluble to 3 per cent.

- 11.32. Can be placed on side.
- 11.34. Moves eyes and head when approached.
- 11.40. When tail is pressed, raises head and turns on belly. Moves head and looks around.
- 11.57. Hops when tail is pressed.
- 12.13. Hops around voluntarily, watching other rabbits. Remains well.
- Rabbit B.*—Oct. 2, 1913. Magnesium sulphate and sodium oxalate. Grey male. Weight 1,720 gm.
- 11.13. Right lumbar muscles: magnesium sulphate  $m$ , 4.8 cc. = 0.7 gm. per kilo. Left lumbar muscles: sodium oxalate 3 per cent, 5.6 cc. = 0.10 gm. per kilo.
- 11.24. Lying prone, with head on floor. When pushed over on side, lies passive. Respiration full; 17 in  $\frac{1}{4}$  min. No response to pressure on tail; lid reflex good.
- 11.38. Same position. Lid reflex hardly perceptible.
- 12.01. Respiration slow and deep; 13 in  $\frac{1}{4}$  min.
- 12.12. No response to stimuli, no lid reflex.
- 12.37. Turns over and lies on belly. Head sinks to floor and is raised at intervals. No response to pressure on tail.
- 1.08. Crouching in a corner. Recovers.
- Rabbit C.*—Oct. 2, 1913. Sodium oxalate alone. Grey male. Weight 1,480 gm.
- 11.21. Left lumbar muscles: sodium oxalate 3 per cent, 4.88 cc. = 0.1 gm. per kilo of body weight.
- 11.28. Sitting up, alert, changes position frequently. Starts suddenly without apparent cause.
- 11.43. Active, hops around, laps water, licks site of injection.
- 12.14. Has been behaving normally. Does not remain in one position long. No further effects noted.

The contrast in the behavior of the controls and the experimental animal is striking. The control animals were but little affected: this dose of sodium oxalate produced only trivial symptoms in Rabbit C, and the magnesium animal, Rabbit A, while weak and stupid, was at no time paralyzed or anesthetic. The third rabbit, however, ten minutes after the injections, was deeply anesthetized and remained passive and insensible for an hour. Two points are of particular interest: (1) In spite of the depth and long duration of the narcosis the respiration continued of good volume and rate and the animal was at no time in danger. (2) The animal regained power of voluntary movement before the return of sensibility to painful stimuli. A series of experiments with similar doses is given in Table I.

TABLE I.

*Magnesium Sulphate and Sodium Oxalate, Intramuscularly.*

No. of experiments.	Dose per kilo of body weight.		Average duration of deep inhibition. Animal relaxed on side.	Died.	Remarks.
	Magnesium sulphate.	Sodium oxalate.			
	gm.	gm.	min.		
6	0.7	0.10	89	0	
6	0.7	—	10	0	Only two out of six relaxed at all.
4	—	0.10	0	0	Visible effect questionable.

*Subcutaneous Injections.*

When the injections were made subcutaneously, somewhat larger doses had to be employed. A typical protocol of an experiment follows.

*Experiment II.*

*Rabbit A.*—Oct. 9, 1913. Magnesium sulphate alone. Slate colored female. Weight 2,030 gm.

10.16. Left flank, subcutaneously: magnesium sulphate *M*, 6.5 cc. = 0.8 gm. per kilo. Massage for 20 seconds.

10.31. Lying down, head and ears erect, breathing rapidly.

10.47. When disturbed hops away clumsily.

11.15. Crouching quietly, head up, ears flat on back. Respiration good.

11.50. Raises head to sniff at nearby objects.

12.20. Sitting up, washing paws. Remains well.

*Rabbit B.*—Oct. 9, 1913. Magnesium sulphate and sodium oxalate. Grey male. Weight 1,755 gm.

10.18. Right flank, subcutaneously: magnesium sulphate *M*, 5.6 = 0.8 gm. per kilo. Left flank, subcutaneously: sodium oxalate 3 per cent, 8.75 cc. = 0.15 gm. per kilo. Both sides massaged for 20 seconds.

10.53. Sitting up naturally.

11.04. Lying with chin on floor. Respiration slower and deep; 21 in  $\frac{1}{2}$  min.

11.13. Placed passively on side without a struggle. Respiration 18 in  $\frac{1}{2}$  min.

11.37. No response to pressing tail. Respiration shallow.

12.50. Trace of lid reflex. No response to pressing tail. Respiration of fair depth; 14 in  $\frac{1}{2}$  min.

2.35. No lid reflex. No response to pressing tail.

3.52. Animal lying as before. No response to stimuli. Breathing entirely abdominal, of fair depth; 14 in  $\frac{1}{2}$  min.

4.28. Does not resist handling. Voluntarily moves head, tail, and legs slightly. Observation discontinued.

Oct. 10, 1913. 9.15. Sitting up in cage. Rather quiet.

*Rabbit C.*—Oct. 9, 1913. Sodium oxalate alone. Grey female. Weight 1,510 gm.

10.22. Left flank, subcutaneously: sodium oxalate 3 per cent, 7.5 cc. = 0.15 gm. per kilo. Massage for 20 seconds.

10.43. Hops around licking the floor and sniffing at objects.

10.53. Sitting up, behaving normally.

11.14. Hops off actively when approached. No effects noted from injection.

Here again neither the oxalate nor the magnesium alone was effective. Together they produced a profound depression with a period of anesthesia and paralysis lasting more than four hours, followed by a gradual complete recovery.

Table II summarizes experiments with subcutaneous injections.

TABLE II.

*Magnesium Sulphate and Sodium Oxalate, Subcutaneously.*

No. of experiments.	Dose per kilo of body weight.		Average duration of deep inhibition. Animal relaxed on side.	Died.	Remarks.
	Magnesium sulphate.	Sodium oxalate.			
	gm.	gm.	min.		
8	0.8	0.15	123 +++	1	In four animals anesthesia extended into the night following. One died next day without recovery.
8	0.8	—	0	0	Practically no effect. Drowsiness in four cases.
8	—	0.15	0	0	No effects observable.

The cited protocols and the two tables illustrate the results obtained in these series of experiments. With the exception of two failures at the beginning, before the proper relation of dosage was determined, the experimental animal in every instance was definitely more deeply affected than the controls. The differences between the various experiments were only of degree, and depended upon the relation of the dose employed and the mode of administration, whether subcutaneous or intramuscular. With proper dose the

contrasts were striking and constant; while the controls were hardly visibly affected, the experimental animals were deeply anesthetized and paralyzed, the character of this inhibition being in general similar to that caused by large effective doses of magnesium alone.

In the following particulars the depression of the animals which received sodium oxalate and magnesium sulphate seemed to differ from that of animals which received magnesium alone. (1) The period of anesthesia and paralysis is a fairly long one, especially after subcutaneous injections, when the state of inhibition may last even 4 hours and longer; whereas after an effective sublethal dose of magnesium alone the entire state of depression is of a comparatively short duration. (2) In animals which receive oxalate and magnesium the deepest stage of anesthesia and paralysis tends to become stationary and is of long duration—the inhibitory curve has a long plateau—and the recovery takes place gradually; whereas with magnesium alone the inhibitory curve has hardly any plateau, and the animal after reaching the acme of anesthesia and paralysis either recovers quite rapidly or the depression leads to death by respiratory paralysis.

The increase of depression following the injection of subminimal doses of sodium oxalate and magnesium sulphate which was definitely established in these experiments cannot be considered simply as a summation of two similar effects. The symptoms brought on by oxalates are entirely dissimilar to those of magnesium inhibition. In our experiments the symptoms which follow the injections of sodium oxalate in toxic doses exhibit the character of excitation; anxiety, restlessness, hypersensitiveness, and tonic and clonic convulsions, which finally lead up to asphyxia and to a fatal termination. In such subminimal doses as we have employed, the toxic symptoms, if there were any, consisted at most in excitation and increased alertness; but there was never any manifest depression. It seems, therefore, that the strikingly depressing effect which the addition of a practically non-toxic dose of sodium oxalate to a subminimal dose of magnesium produces, must be ascribed to the ability of the oxalate to precipitate calcium from the body fluids and thus eliminate an element which biologically is antagonistic to magnesium.

*The Action upon the Motor Nerve Endings.*

Among the general effects of magnesium salts their depressing action upon the motor nerve endings stands out prominently. In minimal effective doses these salts reduce and in larger doses they completely abolish the conductivity of the nerve endings. In a series of experiments we have studied directly the combined action of sodium oxalate and magnesium sulphate upon this intermediary link between nerve and muscle. The sciatic nerve was cut under ether, the animal permitted to recover completely, and then the motor reactions of foot and toes to faradic stimulations of the peripheral end of the sciatic nerve were studied under the influence of the salts under discussion. Seventeen experiments were made upon rabbits. In fifteen there were two rabbits to each experiment, one an experimental animal and one a control. The experimental animals received subcutaneous injections of 0.6 to 0.8 gm. of magnesium sulphate in one side and 0.15 to 0.2 gm. of sodium oxalate. The fifteen control animals received injections of 0.6 to 0.8 gm. of magnesium sulphate alone. Two rabbits received injections of 0.15 and 0.2 gm. of sodium oxalate alone. For faradic stimulations a Porter induction coil, armed with one Daniell cell, was used. The cut sciatic nerve was stimulated before and at various intervals after the injection of the salt solutions, and the degree of the reactions to the various strengths of stimuli was noted. The results obtained in the experimental and control animals were compared and brought into relation with the general condition of the respective animals.

In both the sodium oxalate animals stimulation of the sciatic nerve before and at various times after the injection gave prompt reactions; strong tetanic flexion of the foot and abduction of the toes.

In eight of the magnesium controls stimulation of the sciatic nerve gave normal responses at the various periods after the injection. In the seven other controls there were slight degrees of reduction in the response to the stimulations; the reaction was less prompt, the extent of the contractions was lessened, or the distance of the secondary coil, in order to be effective, had to be shortened.

Of the experimental animals, in thirteen the conductivity of the peripheral nerve endings was definitely more deeply affected than in



their controls. In some cases the conductivity was so depressed that at the time when the general narcosis was at its height no response could be obtained from the stimulation of the sciatic nerve even with a 40 mm. coil distance. In two of the experimental animals the reduction in the response to stimulation of the sciatic nerve was not greater than that of their controls, although the general signs of anesthesia in the experimental animal were quite deep.

The depressing effect upon the motor nerve endings never outlasted the central effects, while there were cases in which the loss of sensation still continued after the motility seemed to be normal again.

### *The Antagonistic Action of Calcium.*

Calcium, as stated in the introduction, is biologically antagonistic to magnesium, and our present experimental results led us to the conclusion that the increase of the depressive action of subminimal doses of magnesium by the addition of a subtoxic dose of sodium oxalate was due to the calcium-precipitating property of this salt. On the other hand, we found that the anesthesia and paralysis produced by a combination of subminimal doses of the two salts was of much longer duration than the same condition produced by an effective dose of magnesium sulphate alone. The question presented itself: Would calcium cause a recovery from the profound long-lasting state of depression caused by the combined action of the two salts, and especially would the recovery be as prompt and as rapid as in cases of magnesium anesthesia? We made a large number of experiments, but our results may be presented in the following single sentence: The antagonistic action of calcium is just as striking and prompt in the prolonged anesthesia brought about by the combination of oxalate and magnesium as it is in the anesthesia produced by magnesium alone. The following protocol is typical for all experiments in this series, and the photographs (Figs. 1 and 2) taken of this experiment are a good illustration of the results.

### *Experiment III.*

*Rabbit I.*—Mar. 9, 1914. Magnesium sulphate alone. Grey and white male. Weight 1,860 gm.

1.58. Right back, subcutaneously: magnesium sulphate  $\text{M}$ , 5.9 cc. = 0.8 gm. per kilo. Massage for 1 min.

- 2.16. Sits quietly in corner of box, or lies down.
- 2.23. Crouching on forepaws, head and ears up. Respiration fair volume, slow; 19 in  $\frac{1}{4}$  min.
- 2.32. Lying at full length, head and ears up. Backs up into sitting posture; rather heavy and quiet.
- 2.47. Crouching quietly in corner of box. Respiration full volume; 14 in  $\frac{1}{4}$  min.
- 3.07. Photographed (Fig. 1).
- 3.16. Photographed (Fig. 2).
- 3.45. Behaving normally and has shown no further effects. Remains well.
- Rabbit II.*—Mar. 9, 1914. Magnesium sulphate and sodium oxalate. Black and white female. Weight 1,540 gm.
- 2.01. Right back, subcutaneously: magnesium sulphate  $M$ , 4.9 cc. = 0.8 gm. per kilo.
- 2.03. Left back, subcutaneously: sodium oxalate 3 per cent, 7.7 cc. = 0.15 gm. per kilo. Massage both sides for 1 min.
- 2.10. Has defecated. Respiration rapid and rather deep. Restless, changes position often.
- 2.13. Hind legs dragged a little in walking.
- 2.21. Crouching, head up, ears back, breathing rapidly; 68 in  $\frac{1}{4}$  min.
- 2.35. Lying full length, eyes half closed, ears back, chin on floor. Flanks relaxed and bulging. Respiration 50 in  $\frac{1}{4}$  min.
- 2.49. Lying partly on side, relaxed, head flat on floor. Mere trace of lid reflex. Moves head slightly when tail is touched.
- 3.05. Placed passively on back, feet in air. Remains there relaxed.
- 3.07. Photographed with controls (Fig. 1).
- 3.15. Same condition. Given 8 cc. calcium chloride 0.125  $M$  through left ear vein. Respiration deepens during injection, and before it is completed animal turns over and sits up.
- 3.16. Photograph taken within 1 minute of injection (Fig. 2).
- 3.45. Crouching quietly. Hair erect. Hops off actively when disturbed. Then sits up with head and ears up. Remains well.
- Rabbit III.*—Mar. 9, 1914. Sodium oxalate alone. White female. Weight 1,620 gm.
- 2.06. Left back, subcutaneously: sodium oxalate 3 per cent, 8.1 cc. = 0.15 gm. per kilo. Massage for 1 min.
- 2.11. Hind legs dragged a little at times. Rather restless.
- 2.30. Sitting up or hopping around naturally. Head and ears up. Not restless or anxious. Respiration 42 in  $\frac{1}{4}$  min.
- 2.48. Behaving normally. Sitting up, quiet. Respiration 35 in  $\frac{1}{4}$  min.
- 3.07. Photographed (Fig. 1).
- 3.16. Photographed (Fig. 2).
- 3.45. Has shown no further effects.

Figs. 1 and 2 illustrate, in the first place, the anesthesia and paralysis produced by the combination of subminimal doses of magnesium and oxalate. They show, further, in a striking way, the antagonistic action of intravenous injection of calcium; it is in all respects similar to the action of calcium in anesthesia by magnesium alone. The respiration becomes deeper and more rapid immediately after beginning the injection, and the return of muscle tone and motor activity can be felt under the hand. Within a minute after the beginning of the injection, often indeed before all of the 8 or 10 cc. of solution is given, the animal draws up its legs, raises its head, turns over and scrambles into a sitting posture, and becomes alert and inquisitive. After an interval the rabbit may gradually sink back into narcosis, and can be restored again by calcium. Occasionally, if too much magnesium and oxalate have been given, a third injection may still be needed and given with success. However, under such circumstances, repeated injections of calcium might finally prove fatal to the animal.

The experiments, showing the depressing effect of magnesium and the antagonistic action of calcium to this depression, are, as we had occasion to learn, frequently demonstrated in many European Universities in lectures on pharmacology or physiology. When magnesium alone is used, the period of the greatest depression is of short duration and the demonstration may either be unconvincing, when the animal is not yet sufficiently narcotized, or it may be a failure, when the calcium injected is administered too late. The anesthesia and paralysis brought about by a combination of sodium oxalate and magnesium sulphate is, as we have seen above, of comparatively long duration. It is therefore a more appropriate method for purposes of demonstration. The animal may receive its double injection 40 to 50 minutes before the time set for the demonstration. If the proper doses are given and the proper procedure is followed out, there is no danger that the animal will not be in deep anesthesia, or that it will die too soon, before the antagonistic effect of the calcium can be shown.

## SUMMARY.

The foregoing experiments establish firmly the following facts.

Subcutaneous or intramuscular injections of sodium oxalate in sub-toxic doses, when administered to an animal which received a sub-minimal dose of magnesium sulphate, produce profound anesthesia and paralysis of long duration, although the usual effects of sodium oxalate alone are of a stimulating character. This fact is, in general, in harmony with the results reported by Starkenstein who, however, seems to have used the combination of the two salts in one solution; namely, that of magnesium oxalate.

The combined injections of subminimal doses of sodium oxalate and magnesium sulphate produce a strong reduction, or even, at times, a complete abolition of the conductivity of the motor nerve endings.

An intravenous injection of calcium salts brings on a recovery from the profound and prolonged effects of the combined action of sodium oxalate and magnesium sulphate, which is as prompt as is observed in experiments in which effective doses of magnesium alone were given. This fact is the more noteworthy, since depressions of long duration produced by prolonged continuous injections of magnesium solutions alone do not respond very promptly and effectively to calcium injections.

As will be recalled, the starting point for our investigation was the hypothesis that substances which are capable of precipitating calcium—a biological antagonist of magnesium—ought to be capable of increasing the depressive effect of magnesium. Our experiments proved that this assumption was correct. This would seem, therefore, to justify the interpretation that the augmenting action of sodium oxalate has its cause in the ability of the latter to precipitate calcium and thus increase within the body the amount of unantagonized magnesium. However, we wish to state expressly that this view is, for the present, still no more than a hypothesis and does not exclude other possible interpretations of our facts. As we pointed out it speaks against this hypothesis that oxalates do not produce phenomena of depression; the toxic symptoms produced by oxalates exhibit distinctly signs of increased and not of decreased irritability.

## EXPLANATION OF PLATE 95.

FIG. 1. Rabbit III. Sodium oxalate 3 per cent, 0.15 gm. per kilo. Alert, ears erect. (Caught by instantaneous exposure.) Rabbit II. Sodium oxalate 3 per cent, 0.15 gm. per kilo. Magnesium sulphate  $M$ , 0.8 gm. per kilo. Deeply anesthetized and quite relaxed. Rabbit I. Magnesium sulphate  $M$ , 0.8 gm. per kilo. Crouches quietly as placed. Ears back.

FIG. 2. Rabbits III and I as before. Rabbit II within a minute has received 8 cc. of calcium chloride 0.125  $M$  into the marginal ear vein (note clip). Alert and sensitive; right paw blurred from movement.



### III

II

I

**FIG. 1.**

### III

II

# I

**FIG. 2.**

(Gates and Meltzer: Action of Sodium Oxalate, Magnesium, and Calcium Salts.)





# THE DISTRIBUTION OF TRYPAN-RED TO THE TISSUES AND VESSELS OF THE EYE AS INFLUENCED BY CONGESTION AND EARLY INFLAMMATION.

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It is the purpose of this paper to present certain simple observations which have a bearing on the manner in which the constituents of the aqueous humor are secreted into and eliminated from the anterior chamber of the eye. It will be shown that external influences may affect profoundly the ease with which abnormal constituents appear in the anterior chamber fluid. The observations have some bearing on general problems of pathology and pharmacology which will be pointed out. And lastly there will be included some observations on the diffusion of substances into and from the cornea.

All the observations have been made on the rabbit's eye. The azo-dye trypan-red as furnished by Gröbler has been the substance chiefly employed for testing the permeability. This dye has been dissolved in normal saline solution to the amount of one-quarter of 1 per cent. 100 cc. of this solution may be warmed to body temperature and injected slowly into a full grown rabbit by the intravenous method without causing the animal any immediate distress and with no appreciable evidence of toxicity later. Under these circumstances by the time the injection is finished the skin and mucous membranes of the entire body are stained red. The intensity of the stain in the tissues increases for a number of hours to a maximum which is maintained without appreciable change for a number of days, and which then gradually fades out over a period of weeks and months. Trypan-blue and other azo-dyes of similar physiological activity may be used in the same general way.

Trypan-red is a colloidal substance; that is, its watery solutions do not diffuse through parchment paper. When, therefore, the dye leaves the blood vessels to appear in the tissues, the lymphatic spaces, lachrymal secretions, or the urine, its passage is in itself evidence that the interposed tissue surfaces are not perfect dialyzing membranes. They are either leaky mechanically to substances of certain physical constitution, or they exert a selective action which permits some colloids to pass while retaining others.

If the normal eyes are observed at any time after the injection of the dye in the manner described it will be seen that the sclera shares the stain of the skin with greater or less intensity. No stain can be detected in the cornea or anterior chamber fluid by inspection of the eye. If, however, after a number of hours the aqueous humor be withdrawn it will be found to have a barely appreciable pink color. If at the end of a week or 10 days the animal is killed, the eye removed, and the cornea dissected free, it also will be seen to be stained very faintly although definitely. If the blood be withdrawn immediately after the injection, the serum is found intensely stained with the dye. As the tissue stain increases in intensity, the stain disappears gradually from the blood to a minimum point which is probably long maintained.

#### *Observations on the Aqueous Humor.*

If within a few minutes after finishing the intravenous injection one eye is cocainized and the anterior chamber fluid withdrawn, a colorless fluid is obtained. As the chamber refills, which it does in the course of a short time, the reformed fluid is stained intensely. The rapidity with which a stained fluid appears in the anterior chamber following such a tap varies considerably in different rabbits. It is also possible so to alter the physiological condition of the eye that the dye when injected intravenously will quickly appear in the anterior chamber without the preliminary tapping.

With the facts above outlined as a basis for work, experiments have been carried out which throw some light on two distinct questions; namely, the place from which certain abnormal constituents may be secreted into the anterior chamber of the eye, and secondly the

relation between the different constituents of an altered anterior chamber fluid in the time of their appearance.

There is now a general agreement that the normal anterior chamber fluid is furnished from the vascularized ciliary body behind the iris, reaching the anterior chamber through the pupil. No experiment entirely free from objection has ever been devised either to prove or disprove this. The experiments in proof of the proposition have involved the withdrawal of fluid from the eye, a procedure which at once disturbs the relationships to an important degree. The experiments of Ehrlich<sup>1</sup> with fluorescin were interpreted by him to signify that the anterior chamber fluid was secreted from particular regions of the anterior surface of the iris. Ehrlich's experiments, done many years ago in part, show that fluorescin appears in the anterior chamber fluid from regions anterior to the iris. It should be recognized, however, that in interpreting such an experiment the fluid and the various other elements of either a normal or an altered aqueous humor may originate in different places. Those portions of Ehrlich's experiments which seemed to him to show that there were definite currents in the aqueous humor flowing from the sides toward the center, meeting on a vertical line in the midregion of the chamber in such a way as to form a swirl where the two streams meet, are susceptible of no explanation in the light of the conditions, as displayed by our work with trypan-red.

When the aqueous humor is withdrawn the pupil usually contracts more or less. Almost immediately the chamber begins to fill again with fluid. When the tap has followed shortly after the intravenous injection of trypan-red, the reformed fluid, as has been said before, is colored with the dye. The color comes for the most part through the pupil from behind. Occasionally it can be seen that the dye makes its appearance in considerable concentration on the anterior surface of the iris away from the pupillary margin before any color has appeared through the pupil.

If eserine is dropped in one eye in sufficient quantity to contract the pupil as far as possible, and if then the dye is injected intravenously, even though no aqueous humor is withdrawn the fluid will

<sup>1</sup> Ehrlich, P., Ueber provocernte Fluorescenzerscheinungen am Auge, *Deutsch. med. Wchnschr.*, 1882, viii, 21.

frequently become colored. Here again the color always appears from behind the pupil, but it may also appear on the anterior surface of the iris independently.

Neither cocaine nor atropine causes the appearance of color in the untapped eye. Atropine when used in such a way as to dilate the pupil fully before the eye is tapped prevents any appearance of dye on the anterior surface of the iris.

If one eye of a rabbit is cocainized and the cornea inoculated with a living culture of the tubercle bacillus, a progressive lesion results, differing in character with the particular culture used and the amount inoculated. The characteristics of this lesion we have described in detail elsewhere.<sup>2</sup> 24 hours after the inoculation there is usually an intense congestion of the conjunctiva, the iris and the ciliary body being more or less congested. This congestion tends to subside by the 2nd day but does not, as a rule, entirely disappear.

24 hours or more after such an inoculation, if the animal is injected with the dye intravenously as above described, the anterior chamber fluid of the inoculated, untapped eye will always become colored. Here again most of the color appears through the pupil, but in many instances it also appears earlier and independently on the anterior surface of the iris.

In each of these instances, the tapped eye, eserinated eye, or the tubercular eye, whenever the color appears on the anterior surface of the iris, it seems to be associated with definite areas of congestion. It would be interesting to know whether it is impossible to have the color appear abnormally in the absence of such a congestion. We have, with this in mind made some experiments with abrin. This poison, as is well known, causes an intense inflammation of the conjunctiva when it is dropped in the eye. The inflammation is characterized by a well marked edema and congestion and develops slowly through a stage which is, generally speaking, one of edema, to a stage which is more predominantly congestive. In the various stages of this inflammation trypan-red or trypan-blue applied intravenously appears in the anterior chamber, and in the edematous conjunctiva with unusual rapidity. We have never had a result, however, which

<sup>2</sup> Lewis, P. A., and Montgomery, C. M., *Jour. Exper. Med.*, 1914, **xx**, 269.

enables us to think that the dye can appear in abnormal amount or situation as a consequence of an edematous condition alone and in the entire absence of congestion.

To sum up this portion of our remarks we may say that under conditions in which the eye is slightly congested certain dyes injected intravenously may appear in abnormally large amount and with unusual rapidity in the aqueous humor. The largest amount of the dye comes into the aqueous humor from behind the iris, but appreciable amounts frequently come from the anterior surface of the iris. In these instances the iris has always shown a local congestion in the region in which the dye has appeared.

It is a well known fact that if the aqueous humor be withdrawn the fluid which refills the anterior chamber differs from that first withdrawn in that it coagulates spontaneously. We have tested the relationship between the appearance in the fluid of the factors controlling the coagulation and the dye. When the dye is injected intravenously, the eye being tapped at once after the injection is finished, the rapidity with which the dye appears in the anterior chamber varies greatly in different rabbits. It is usually from 20 to 30 minutes before fluid is obtained which would be classified as intensely stained. At this time the fluid has always coagulated spontaneously in our experience. Occasionally we have had animals in which at the end of 5 minutes the anterior chamber fluid was intensely stained. On withdrawing this at once we have several times obtained an intensely colored fluid which did not coagulate. These facts suffice to show that the appearance of the dye is independent of the appearance of at least some of the factors determining coagulation.

The distribution of the dye when it comes into the anterior chamber is interesting in that it throws some light on the rapidity of movement of the aqueous humor. In those instances where the dye appears in concentration on the iris surface before it comes through the pupil it diffuses very slowly from the point where it makes its appearance, and this diffusion is apt to be more or less even in all directions. When the dye comes through the pupil it is apt to come over the lower pupillary margin in a concentrated stream which sinks slowly to the bottom of the chamber and from there, spreads in the course of half an hour or more by diffusion until the aqueous is evenly colored.

The conditions can easily be duplicated outside the body. If one takes a thin collodion sac about an inch in diameter, fills it with a 1 per cent solution of trypan-red, and gently lowers it into a beaker of water the dye will diffuse out of the sac into the water with moderate rapidity. The dye comes out of the sac over the entire submerged surface. It then apparently creeps along the surface of the sac to the lower end of it and falls from there in a narrow stream of concentrated dye to the bottom of the beaker. It spreads along the bottom to make a concentrated red layer in this region. Then in the course of an hour or more it diffuses throughout the water. If the beaker is disturbed, of course the secondary spread is hastened.

The conditions in the anterior chamber of the eye are analogous to this, and it is hard to reconcile the observations with the view that there are any very active movements in the aqueous humor. There seems to be a tendency for matter coming into the anterior chamber through the pupil, to come in at the lower margin and to sink to the bottom of the chamber.

There is also a tendency for the dyes that we have used, at least, to leave the chamber in a definite region. If 0.5 cc. of a 1 per cent solution of trypan-red or trypan-blue is taken in a suitable syringe and the aqueous humor from a normal eye is also drawn into the same syringe and if then the mixed fluid and dye solution is immediately reinjected, care being taken to restore approximately the original tension, the absorption of dye can be watched very well. Within a few minutes vessels in the sclerotic will be seen to be injected with the dye. The vessels which are first injected and the only ones which usually show a pronounced injection are on the upper surface of the eyeball to each side of the midline. The injection of vessels in this region persists until the dye is completely absorbed.

The indication in these experiments is, then, that there is very little movement in the aqueous humor. The points of secretion and absorption are such as to determine fairly definite lines of diffusion for colloidal matter in the fluid, which in a general way are from behind the iris, through the pupil to the lower portion of the chamber, and then upward to leave at the superior portions of the angle.

*Observations on the Cornea.*

In a preceding paragraph we said that a number of days after an intravenous injection of trypan-red the cornea becomes distinctly colored. It is the generally accepted view that substances which reach the cornea do so by diffusion from the corneoscleral margin. The way in which the cornea becomes stained is in accord with this. If an animal is killed 2 or 3 days after the dye is injected, the cornea will, on examination, be found stained at its circumference, the colored area at this time reaching about one-third of the way to the center.

In connection with the study of experimental tuberculosis previously referred to, we observed that the reactions of the cornea were not uniform throughout. If, for example, a central inoculation is made, the first formation of blood vessels at the corneoscleral margin will be above, at approximately the midline. Next, vessels will form on the midline below, and, lastly, on the sides. We assumed that this was because substances diffused out of the cornea by preference toward the upper portion and hence stimulated the tissue reactions first at that point. That the diffusion is along these lines we have now found can be shown to be the case with the dyes we have used in this work. If the cornea is infiltrated in a spot 2 to 3 mm. in diameter at its center, the dye does not diffuse toward the lower corneoscleral margin in any appreciable degree. The diffusion is chiefly toward the upper margin, spreading out more or less in the shape of a fan in this direction. To get a diffusion chiefly toward the sides or lower margin it is necessary to place the infiltration quite close to the corneoscleral junction in those directions.

**SUMMARY.**

In as far as the observations reported have a bearing on the movements of fluid within the eye, they are, for the most part, in accord with views at present generally accepted. On the other hand, we know of no other way in which it may be so readily demonstrated that simple and even temporary local circulatory changes may profoundly alter the distribution of substances from the circulating

blood to the extravascular fluids and tissues. In the light of these observations, it would seem that such changes might easily account for marked idiosyncrasies in the action of poisonous drugs, and as well probably for other factors in drug action.



# THE APPEARANCE OF THE PRESSOR SUBSTANCE IN THE FETAL HYPOPHYSIS.

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PLATES 96 AND 97.

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It has been demonstrated (Lewis, Miller, and Matthews<sup>1</sup>) that the pressor substance of the posterior lobe of the hypophysis is secreted by the pars intermedia, a derivative of the pharyngeal pouch. The present study was undertaken with the hope of determining at what period in fetal life the pressor substance appears and of correlating the cytological changes in the pars intermedia with the establishment of secretory function, using the appearance of the pressor substance as an index. The cytological studies are now in progress.

I have used extracts from the entire gland. The hypophyses of pigs just before birth are large enough to permit of separation of the two lobes, but in the earlier stages this is impossible. In order to secure uniform material for injection the extracts were made from the entire gland in all instances. The glands were obtained fresh and extracted in absolute alcohol to remove the depressor substances. After extraction was completed, the alcohol was filtered off and the residue dried in a desiccator. The dried residue was kept in small bottles until desired for use, when a salt solution extract was made for intravenous injection.

Fetal pigs just before birth measure from 275 to 285 mm.; the measurement being taken from the crown of the head to the tip of the coccyx. Fig. 1 indicates the effect upon blood pressure of an intravenous injection of 0.1294 gm. of dried hypophyses removed from pigs just before birth, extracted in 5 cc. of normal salt solution.

<sup>1</sup> Lewis, D., Miller J. L., and Matthews, S. A., The Effects on Blood-Pressure of Intravenous Injections of Extracts of the Various Anatomical Components of the Hypophysis, *Arch. Int. Med.*, 1911, vii, 785.

This extract must be carefully filtered before injection, for much of the dried glands is insoluble. Intravenous injection of 5 cc. of this extract caused a prompt and marked rise in blood pressure which was followed by a fall and a second rise to almost the same level as the first, accompanied by marked slowing of the pulse and an increase in the length of the pulse wave. This type of tracing is often obtained after intravenous injections of extracts made from adult glands, but it is not the most common type of tracing obtained after such injections. It has been suggested that the fall following the primary rise, which is of short duration, is probably due to coronary artery contraction. This type of tracing seems to occur more frequently when extracts of the entire posterior lobe are used than when those of the pars intermedia alone are employed. It has been suggested that the variations from the common type which will be noted later may be due to the action of substances extracted from the nervous part of the posterior lobe.

Intravenous injection of 0.05 gm. of dried hypophyses from fetal pigs measuring 175 mm. extracted in 5 cc. of normal salt solution gave a decided pressor effect such as is usually obtained by the intravenous injection of extracts made from adult glands. The pressor reaction followed the injection rapidly and was long continued, being unassociated with any marked change in the tracing (Fig. 2).

Intravenous injections of saline extracts of hypophyses from fetal pigs measuring 125 mm. gave different results. In some cases a slight pressor effect following a primary fall was noted, while in other instances no change in the tracing was noted. Fig. 3 is a tracing obtained after intravenous injection of an extract of 0.08 gm. of dried hypophyses in 5 cc. of normal salt solution. The injection of this extract caused a fall in pressure which was followed by a rise slightly above the level existing before the injection was made. This reaction was of short duration, the pressure soon sinking to the level which existed before the injection was made. Another tracing was made after the injection of an extract of 0.06 gm. of dried hypophyses from pigs measuring 125 mm. extracted in 3 cc. of salt solution. The amount of extract injected was therefore practically the same as in the preceding experiment. The injection of this extract caused a fall in pressure followed by a rapid return to the

level existing before the injection was made (Fig. 4). An injection of the residue left after filtration of the preceding solution extracted in 5 cc. of normal salt solution caused another fall with a rapid return to the level existing before the injection. The fall in pressure observed in the last experiments occurs frequently after injections of glands, even after attempts to remove completely the depressor substance which exists in all parts of the gland have been made by thorough extraction with absolute alcohol.

Fig. 3 indicates that in some instances a slight but unmistakable pressor effect of short duration may follow the intravenous injection of extracts made from the hypophyses of pigs measuring 125 mm. This reaction does not follow the injection of all extracts made from the glands of pigs of this measurement. It is possible that in attempts to obtain fresh material a few pigs measuring slightly more than 125 mm. have been occasionally used, but if this is the case, they have exceeded this measurement only by a few mm.

I believe that I am justified in stating that the active principle of the pars intermedia, using the pressor substance as an index, begins to appear in fetal pigs measuring 125 mm. or slightly more.

It is somewhat difficult to determine the age of fetal pigs as estimated by measurements. Koch says that great difficulty is experienced in finding any statement concerning the age of pig fetuses. The statements of different authors do not always agree, but the two which come closest to an agreement are those of Bradley and Coe. Bradley compared the length of the embryos with the time of coition. Coe estimated the age from the rate of development of other mammals. While considerable uncertainty is attached to these figures, it may be assumed that the 50 mm. pig fetus is about 40 days old from conception; the 100 mm. fetus is 55 to 62 days old; and the 200 mm. pig is 88 to 90 days old from conception.

As determined by the pressor reaction the secretion of the pars intermedia seems to be established in pigs measuring 125 mm. The reaction is not obtained after the injection of all extracts of glands removed at this period and even when present is not marked. There are, however, often definite indications of a pressor effect as indicated in Fig. 3. As far as can be estimated, a pig measuring 125 mm. is about  $9\frac{1}{2}$  or 10 weeks old from conception. During the period rep-

resented by the differences between 125 and 175 mm., the secretion of the pars intermedia becomes as active as that of the adult gland.

McCord<sup>2</sup> has recently attempted to determine the time of appearance of the active principle of the pars intermedia. Its presence was determined by the oxytocic activity of extracts of glands by means of the method of Dale and Laidlaw,<sup>3</sup> using histamine as a standard. The tests for pituitrin were begun with embryos at or near full term. These tests and others as far back as 9 weeks were quantitative tests. Pituitrin was found in the extracts of glands as early as 9 weeks, and the quantity for unit of weight was larger than in the adult. In the 7th and 8th weeks of fetal life the pituitary could no longer be recognized, although the sella turcica was plainly visible. The experiments, therefore, could not be made earlier than the 9th week of fetal life.

McCord worked with bovine fetuses and it is difficult to determine the relative ages of calf and pig fetuses by the lengths. McCord found that the first indication of an active pars intermedia occurs in a bovine fetus measuring 165 mm., while evidences of an active pars intermedia are found in a pig fetus measuring 125 mm.

Fenger<sup>4</sup> has shown that both the thyroid and suprarenal glands of the beef, hog, and sheep contain their active principles not only at birth, but also in the fetus. He believes that both the thyroid and suprarenals of the fetus take a distinct and active part in its growth.

#### SUMMARY.

The pressor substance of the hypophysis is so marked in the pig fetus measuring 175 mm. that it seems probable that a fetus of this length is independent of the secretion of the mother's hypophysis.

<sup>2</sup> McCord, C. P., The Occurrence of Pituitrin and Epinephrin in Fetal Pituitary and Suprarenal Glands, *Jour. Biol. Chem.*, 1915, xxiii, 435.

<sup>3</sup> Dale, H. H., and Laidlaw, P. P., A Method of Standardizing Pituitary (Infundibular) Extracts, *Jour. Pharm. and Exper. Therap.*, 1912-13, iv, 75.

<sup>4</sup> Fenger, F., On the Presence of Active Principles in the Thyroid and Suprarenal Glands Before and After Birth. Second Paper, *Jour. Biol. Chem.*, 1912, xii, 55.

## EXPLANATION OF PLATES.

## PLATE 96.

FIG. 1. Effect upon blood pressure of the intravenous injection of 0.1249 gm. of dried hypophyses removed from pigs just before birth, dissolved in 5 cc. of normal salt solution. This type of tracing is often produced by injection of extracts of the adult gland, but is not the most common form.

FIG. 2. Effect upon blood pressure of the intravenous injection of 0.05 gm. of dried hypophyses removed from pigs measuring 175 mm., dissolved in 5 cc. of normal salt solution. A prompt and long continued pressor effect is noted.

## PLATE 97.

FIG. 3. Effect upon blood pressure of the intravenous injection of 0.08 gm. of dried hypophyses removed from pigs measuring 125 mm., dissolved in 5 cc. of normal salt solution. The fall in pressure is followed by a distinct but temporary rise.

FIG. 4. Effect upon blood pressure of the intravenous injection of 0.06 gm. of dried hypophyses removed from pigs measuring 125 mm., dissolved in 3 cc. of normal salt solution. A fall in pressure is noted with a rise to the level existing before the injection.



FIG. 1

FIG. 2.

(Lewis: Pressor Substance in Fetal Hypophysis.)





## CHEMICAL VERSUS SERUM TREATMENT OF EPIDEMIC MENINGITIS.

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### INTRODUCTION.

The reappearance of epidemic meningitis among the belligerent armies in Europe has led to numerous reports on the treatment of the disease. For the most part, the antimeningococcic serum has been employed, but in one instance at least the attempt has been made to improve upon the serum treatment by substituting for it chemical treatment. This attempt to employ directly antiseptic drugs in the treatment of epidemic meningitis is a revival of an old notion.

The circumstances are such as to make the chemical treatment favorable from a theoretical standpoint. The infection tends to be local, that is, within the cerebrospinal membranes, and not general or throughout the body. The membranes are directly accessible from without, so that the chemical substance can be brought into immediate relation with the seat of infection. Moreover, the meningococcus as cultivated outside the body is not a highly resistant microorganism, but is readily injured by chemical action, and is indeed quickly injured by the waste products of its own growth. What, therefore, is apparently simpler or more promising than to control epidemic meningitis by the direct application by means of lumbar puncture of bactericidal chemicals to the infected and inflamed cerebrospinal membranes?

There is, moreover, still another theoretical reason for resorting to the chemical treatment of epidemic meningitis. The meningococcus is now known to be a microorganism which occurs in at least two varieties or types, distinguished as meningococcus and parameningococcus.<sup>1</sup> Their main differences are immunological, so that they

<sup>1</sup> Amoss, H. L., and Wollstein, M., *Jour. Exper. Med.*, 1916, xxiii, 403.

are subject to therapeutic influences through antisera only when correspondence exists between the infecting type of microorganism and the particular specific antibodies present in a serum. Presumably, chemical agents being less specific would be less restricted in their effects, and might be expected to act quite irrespective of the type of meningococcus causing the infection.

Wolff<sup>2</sup> has recently recommended the employment of protargol in the treatment of epidemic meningitis, basing the recommendation of its use on eight cases, of which five recovered. The point of departure in the selection and administration of protargol was the successful employment of so called colloidal silver preparations in the treatment of gonorrhea, and the close relationships as regards conditions of culture, survival, and reaction to antiseptics subsisting between gonococci and meningococci. It is conceded by Wolff that because of the highly variable clinical course of epidemic meningitis, the number of cases which he reports is too small to permit of the drawing of any conclusions regarding the therapeutic efficiency of the chemical. He believes, however, that it is at least innocuous and can, therefore, be administered with the assurance that if it is not curative, it will do no harm.

The first method of direct chemical treatment of epidemic meningitis to receive considerable attention was that in which lysol was employed. The method was introduced in Lisbon during the epidemic which prevailed there in the first years of the present century, and was briefly reported on by Seager<sup>3</sup> in 1902. Following Seager's report, lysol was tested in the course of the epidemic from which Greater New York was suffering at the time.<sup>4</sup> This epidemic continued for several years and led to the working out of the serum treatment, but at that early date no specific means of combating the disease was known.

It is remarkable and instructive to consider that in these instances, namely, the one relating to lysol and the other to protargol, what appeared to be preliminary success was achieved by the chemical treatment. Wider experience, however, led to the abandonment of

<sup>2</sup> Wolff, G., *Deutsch. med. Wchnschr.*, 1915, xli, 1486.

<sup>3</sup> Seager, H. W., *Lancet*, 1902, ii, 1188.

<sup>4</sup> Manges, M., *Med. News*, 1904, lxxxiv, 913.

the lysol treatment. In the meantime, knowledge has been gained and methods perfected through which the value of a proposed form of treatment of epidemic meningitis can be determined experimentally in animals, with a degree of accuracy exceeding that based on clinical and limited statistical observations on human beings.

It happens that epidemic meningitis in man is a highly variable disease. The severity fluctuates between clinical conditions so slight as to justify the term abortive being applied to certain cases, and at other times so severe (fulminant) as to cause death swiftly, and sometimes with the first appearance of symptoms. Hence the mortality percentage of the disease varies in different epidemics and places. To determine approximately what the effects of varying modes of treatment are it is necessary to compare coincidentally treated and untreated cases of sufficient number. A definite result is therefore obtained only after some time, and by comparing figures obtained in different localities and over a comparatively long period of time. So long as the figures based on small local observations alone are available, no final deduction can be made.

The employment of animals for the test is free of the uncertainty of the statistical method. The dosage of the culture of meningococcus can be adjusted to produce the result desired. Then the effect and the manner of the therapeutic action can be accurately ascertained. Tests of this kind have been made with protargol and for comparison with lysol and with serum, with results unfavorable to the chemical treatment.

#### EXPERIMENTAL.

The experimental studies on epidemic meningitis carried out during the past 10 years have shown two kinds of animals to be suitable for determining the pathogenic action of meningococci and the effects of therapeutic agents in meningococcic infection. The animals are monkeys<sup>5</sup> and young guinea pigs.<sup>6</sup> While the age of the monkeys seems not to matter, older guinea pigs are highly resistant to meningococcic infection. The cultures of meningococci are injected into

<sup>5</sup> Flexner, S., *Jour. Exper. Med.*, 1907, ix, 142.

<sup>6</sup> Flexner, S., *Jour. Exper. Med.*, 1907, ix, 168.

the peritoneal cavity in the guinea pigs and into the subarachnoid space in the monkeys, in the latter by means of lumbar puncture. The injected meningococci set up local inflammations in the exudate attending which the fate of the microorganism can be followed precisely, as in human cases of epidemic meningitis in which the cerebrospinal fluid is removed from time to time by lumbar puncture.

The changes occurring in the meninges or the peritoneum vary with the activity and the dose of the culture. Non-virulent cultures are quickly phagocyted, no considerable multiplication takes place, and the inoculated animals show few or no symptoms and recover fully in 24 hours. Active cultures multiply, are imperfectly phagocyted, and cause death according to the virulence and dose in 6 to 8 to 12 hours in guinea pigs, and in 20 to 48 hours in monkeys. At autopsy living meningococci are present in the peritoneal or meningeal exudate and in the blood. Film preparations show meningococci free and phagocyted also in the peritoneal fluid and more numerous in the omentum of the guinea pig, and both free and phagocyted in the exudate in the pia-arachnoid membranes of the monkey.

In carrying out the experiments a virulent strain of the meningococcus (Isadore) was employed. Tests made to determine the optimum period of growth at which to employ the culture indicated it to be after about 18 hours' incubation at 37°C. Sheep serum dextrose agar was used as a medium. Growths 24 hours old infected far more irregularly than those 18 hours old. The reason for the disparity is found in the spontaneous degeneration of the culture, which probably has already begun at 18 hours, and can be detected by microscopic examination at 22 hours. In the guinea pigs it was found in this instance, as already described,<sup>1</sup> that consistent results are best attained by employing one minimal lethal dose of the culture. In this way, lack of protective power is made to assert itself readily, while the existence of protective power is easily demonstrated and can be further investigated. In monkeys the infecting dose is easily determined, although the fatal dose is established less readily. While these animals were used sparingly, the protocol in each instance indicates the outcome of the therapeutic test compared with the control. Besides the result based on death or survival, the changes

taking place in the cerebrospinal fluid as followed by lumbar puncture, and the clinical course of the experimental disease, to a less definite degree, are also valuable indications of the action of the therapeutic agent.

### *Tests with Guinea Pigs.*

The guinea pigs ranged in weight from 90 to 110 gm. The injections were intraperitoneal. The protargol was employed in 0.2 per cent strength, prepared by suspending the dried powder in sterile distilled water. The cultures of meningococci on sheep serum agar after 18 hours' incubation were suspended in sterile salt solution, and injected either immediately or after such intervals as are indicated in the separate experiments. The dose was one m.l.d.

TABLE I.  
*Experiment 1. Toxicity of Protargol.*

Weight of guinea pig.	Quantity of suspension injected.	Result.
gm.		
90	0.3 cc. + 0.7 cc. water.	Survived.
90	0.5 " + 0.5 " "	"
90	1.0 " + 1.0 " "	"
90	2.0 " + 2.0 " "	Died in 38 hrs.

The tests shown in Table I may be taken to indicate that the toxic dose of protargol for young guinea pigs is well above the dose employed for therapeutic purposes.

*Experiment 2. Therapeutic Tests of Protargol Intraperitoneally in the Guinea Pig.*—One m.l.d. of living culture in a total volume of 1 cc. was injected intraperitoneally into young guinea pigs. The amount of 0.2 per cent protargol suspension given was made up to 1 cc. with sterile water and injected at the time indicated in Table II.

TABLE II.

Weight of guinea pig.	Dose of protargol suspension.	Time of administration.	Result.
gm.	cc.		
110	0.5	Immediately.	Death in 12 hrs.
110	0.5	"	" " 21 "
95	0.5	"	" " 15 "
100	0.5	"	" " 10 "
99	0.1	"	" " 8 "
82	0.1	"	" " 19 "
82	0.1	"	" " 11 "
100	0.5	After 15 min.	" " 12 "
110	0.7	" 15 "	" " 8 "
100	0.5	" 1 hr.	" " 10 "
75	0.1	" 1 "	" " 15 "
75	0.1	" 1 "	" " 18 "
95	0.25	Mixed with culture, injected after 1 hr.'s contact.	" " 25 "
110	1.00	" " "	" " 14 "

This experiment is conclusive in demonstrating that protargol in 0.2 per cent suspension, the strength employed by Wolff,\* is incapable of preventing in guinea pigs the lethal effect of a single minimal fatal dose of the meningococcus, whether administered combined with the culture immediately after the mixture, or whether the culture and protargol are injected separately 15 minutes apart. The period of survival of the treated animals may be about the same as or somewhat greater than the controls. Living meningococci are always found in the peritoneal cavity and in the heart's blood. In this connection it is interesting to find that when the culture and protargol suspension which have been in contact for 1 hour are transplanted to fresh sheep serum agar, no growth is obtained; while, however, cultures from the peritoneal cavity of the dead animals which were inoculated with the mixture are positive, thus indicating that the protargol merely inhibits the growth of the meningococci, but may not destroy them in 1 hour's time. This observation raises the question whether the failure of the protargol is due merely to its imperfect bactericidal power, or whether it depends upon lack of antitoxic power at the same time. The next experiment bears on this consideration.

*Experiment 3. Therapeutic Test of Protargol with Autolysate.*—An autolysate<sup>7</sup> of the meningococcus was prepared in the usual way by suspending the culture in salt solution, adding toluene, incubating, allowing the toluene to evaporate, and centrifugalizing. The clear supernatant fluid was employed. The autolysate and protargol suspensions were mixed and immediately injected, as shown in Table III.

TABLE III.

Weight of guinea pig.	Dose of autolysate.	Dose of suspension of 0.2% protargol.	Result.
gm.			
90	0.25 cc. in 1 cc. salt solution.	None.	Died in 7 hrs.
100	0.25 " " 1 " " "	0.5 cc.	" during night.
110	0.25 " " 1 " " "	1.0 "	" " "

This experiment indicates that protargol does not exhibit pronounced antitoxic properties, although a delay was noted in the fatal issue in the animals in which it was given.

*Mechanism of the Action of Protargol and of Antiserum.*

In carrying out the above tests which were made on several different occasions, control observations with antimeningococcic horse serum were also made. The quantity of serum administered was 0.5 cc., and in every one of the six instances in which the antiserum was given the animal survived. The antiserum was also injected in association with the protargol, without, however, preventing a fatal issue. On the other hand, the antiserum influences directly the mechanism of action of protargol, although unable apparently to overcome wholly some injurious effect which it exerts. The next experiment, therefore, was devised to bring out definitely the mechanism of action of the protargol in comparison with the manner of action of the antiserum.

*Experiment 4. Mechanism of the Action of Protargol.*—Four guinea pigs, ranging in weight from 90 to 110 gm., were employed. Besides the inoculation of meningococci, one of them received immediately 0.5 cc. of a suspension of 0.2 per cent protargol alone; the second received 0.5 cc. of antiserum alone; and

<sup>7</sup> Flexner, S., *Jour. Exper. Med.*, 1907, ix, 105.

the third 0.5 cc. of a suspension of protargol and 0.5 cc. of antiserum in immediate succession. The fourth animal received only the culture. All were chloroformed at the expiration of 6 hours, at which time the control animal was prostrate and the others were definitely ill.

The autopsies were made at once and the distribution of meningococci in the abdominal cavity studied by means of cultures and film preparations, and in the blood of the heart by cultures alone.

*Guinea Pig 1. Protargol Alone.*—Two drops of the blood of the heart were laked in 0.5 cc. of sterile water and plated in sheep serum agar. The colonies numbered about 2,000. Two drops of the slightly turbid peritoneal fluid were similarly plated. The colonies numbered about 2,000. The film preparations from the fluid in the abdominal cavity showed numerous scattered meningococci and a notable absence of polynuclear cells, and the omentum showed scattered free meningococci and polynuclear leukocytes, of which a very small number contained diplococci, the leukocytes themselves staining feebly and appearing degenerated.

*Guinea Pig 2. Antiserum Alone.*—Two drops of the heart's blood were treated as in No. 1. No colonies. Two drops of the somewhat turbid peritoneal fluid gave about 2,500 colonies. Film preparations of the peritoneal fluid showed numerous well preserved polynuclear leukocytes containing many meningococci, while the extracellular diplococci, small in number, were arranged in small clumps. The film preparations from the omentum exhibited many more leukocytes, most of which were filled with meningococci in various stages of disintegration, the leukocytes themselves appearing in normal condition. A few extracellular agglutinated meningococci were also present.

*Guinea Pig 3. Protargol and Antiserum.*—Two drops of the heart's blood yielded one colony, and two drops of the clear peritoneal fluid about 150 colonies. Film preparations of the peritoneal fluid showed a smaller number of diplococci than in either of the preceding animals. Polynuclear leukocytes were present, but in far smaller number than in No. 2, and many were degenerated and had rarely phagocyted the diplococci. Such meningococci as were present at all were agglutinated. The films from the omentum showed somewhat larger numbers of degenerated polynuclear cells, of which a part contained meningococci, and quite numerous free, single and clumped diplococci.

*Guinea Pig 4. Control.*—Two drops of the heart's blood gave about 2,000 colonies, and two drops of the clear peritoneal fluid innumerable colonies. Congestion of the peritoneal organs was more marked in this animal than in the others. Film preparations of the peritoneal fluid showed a very large number of particularly well preserved meningococci, but no polynuclear leukocytes, and those of the omentum showed a similar condition. The absence of polynuclear cells from the latter structure is particularly noteworthy.

This experiment confirms the observation made upon the guinea pigs treated with protargol which were allowed to succumb. Its



significance is clear. Protargol has some power to cause dissolution of the meningococci with which it comes in contact, but does not destroy all. A later increase occurs and invasion of the blood stream takes place. Probably the injurious action of the protargol arises from its antileukotactic properties, for it causes degeneration of the polynuclear leukocytes and prevents phagocytosis, both *in vivo* and *in vitro*.

This particular action of the protargol is rendered obvious at once on comparison with the manner in which the antiserum acts. In this instance there is a diversion of polynuclear leukocytes in large numbers to the peritoneal cavity and an acceleration of phagocytosis. Hence at a period at which many living meningococci are still present in the abdominal cavity, practically no escape is taking place into the general blood stream. The addition of the antiserum to the protargol obviates partially the antileukotactic effect of the latter, but does not completely overcome it, because of the injury inflicted by the chemical on the leukocytes brought out by the antiserum.

Protargol is, therefore, bactericidal for the meningococcus, as for the gonococcus, through direct chemical action, but it is not curative in meningococcic infection in the guinea pig, because being confined within the abdominal cavity it exerts a passive chemotactic and antiphagocytic influence on the polynuclear cells which are themselves injured and destroyed through its action. Thus the imperfect bactericidal effect is inadequate to control infection, while the antileukotactic action is itself harmful and promoting of infection. Moreover, the masses of protargol are themselves taken up by leukocytes, to be transported doubtless to organs in which the substance may remain indefinitely.

#### *Tests with Monkeys.*

The inoculation of virulent meningococci into the subarachnoid space in monkeys causes an acute inflammation which extends throughout the meninges and into the cerebral ventricles. Depending upon the pathogenic power and the dose, the animal which develops symptoms within a few hours may die within 24 hours or may recover. According as one or the other result is obtained, the meningococci multiply and are imperfectly phagocyted, or they increase little

and are perfectly phagocyted. The cerebrospinal fluid at first remains clear, then becomes turbid with emigrated polynuclear leukocytes; finally it may again become clear. A blood invasion often follows within a few hours of the inoculation, but is rarely present at the end, even in fatal cases.

*Experiment 5. Control.*—A *Macacus rhesus* was injected at 11 a.m. intraspinally with 2 cc. of a salt solution suspension, representing 1½ agar slant cultures of meningococcus, the strain being the same as that used in the experiments on the guinea pigs. 5.00 p.m., animal sick. Lumbar puncture yielded a fluid already turbid, giving positive culture and showing on films many meningococci, few leukocytes, and little phagocytosis. Blood taken from arm vein gave positive cultures. 9.00 a.m. the next day, prostrate. Lumbar puncture yielded highly turbid fluid, giving positive cultures and films in which leukocytes were numerous and of which many contained meningococci; free diplococci were also present. Death at 3.30 p.m. Survived 20½ hours.

*Autopsy.*—The membranes of the brain and spinal cord showed turbid fluid and congestion. The cultures and film preparations confirmed previous findings. Both intra- and extracellular meningococci were present. Cultures positive. Cultures of heart's blood negative.

Sections from the brain and spinal cord show an acute fibrinopurulent meningitis, most pronounced over the convex surface of the brain. The ventricles are involved slightly. Many diplococci are present in the exudate and are contained chiefly, if not wholly within leukocytes.

*Experiment 6. Treatment with Antimeningococcic Horse Serum.*—8.30 a.m., a *Macacus rhesus* received intraspinally an injection of a suspension of meningococci as in Experiment 5. 12.30 p.m., lumbar puncture yielded cloudy fluid, giving positive cultures. Films showed numerous meningococci, pus cells, and phagocytosis already present. The culture from the heart's blood was negative. 2 cc. of antiserum were injected. 4.30 p.m., animal appeared well; lumbar puncture yielded turbid fluid, giving positive culture and showing in the film marked phagocytosis and agglutination of all extracellular meningococci. 8.30 p.m., active. 28 hours after the inoculation the animal appeared well; lumbar puncture yielded a turbid fluid which gave a negative culture. The films showed many leukocytes, some containing meningococci and very few extracellular diplococci. A number of endothelial cells and lymphocytes were present. The animal remained well.

*Experiment 7. Treatment with Protargol.*—8.30 a.m., a *Macacus rhesus* received an equal part of a suspension of the culture used in the previous experiments. 12.30 p.m., little change in condition. Lumbar puncture yielded a cloudy fluid, from which cultures were positive and films show many leukocytes and well advanced phagocytosis of meningococci; some free meningococci were also present. Injected 2 cc. of 0.2 per cent protargol suspension. 2.30 p.m., animal

lying down, drowsy, surface cold, limbs spastic. 4.00 p.m., respiration rapid, slight convulsion recurring frequently. Blood withdrawn at this time gave a positive culture. 4.30 p.m., died. Survived 8 hours.

*Autopsy.*—No lesions were found outside the cerebrospinal membranes. The meninges contained an excess of almost clear fluid. No marked congestion. Cultures from the meninges of the brain and spinal cord yielded luxuriant growth. Films showed a large number of meningococci, both intra- and extracellular, and a widespread degeneration and even disintegration of leukocytes. The meningococci stain sharply and deeply. The choroid plexus of the lateral ventricle and the meninges of the olfactory lobes gave results similar to those of the other membranes. Cultures from the heart's blood were positive. Moreover, cultures prepared from the nasal mucosa yielded, among other bacteria, typical meningococci, possessing identical agglutination reactions with the strain employed for inoculation.

The microscopic examination of sections from the central nervous organs shows many pus cells, with here and there red corpuscles, and no fibrin. The number of meningococci present is very large. Some degree of phagocytosis exists; but a striking phenomenon is the packing of lymph spaces with innumerable diplococci. They invade the pial spaces, infiltrate the superficial layers of the cerebral cortex, and follow the perivascular spaces for some distance into the cortex. Certain coagula of red and white corpuscles within the central ventricles also enclose diplococci.

A comparison of the results in the three monkeys inoculated with meningococci and left in one case untreated, and in the others treated either with antiserum or protargol, is unfavorable to the treatment with protargol. The untreated or control animal succumbed under conditions indicating definite if inadequate reaction against and resistance to the infection. The serum-treated animal was already ill when the antiserum was administered, the effect of which was to disperse almost immediately all the symptoms and lead to prompt recovery, coincidently with the removal by phagocytosis of the meningococci. The third monkey exhibited practically no symptoms 4 hours after the inoculation when the protargol was given, but prostration and rapid intensification of symptoms followed almost immediately. Death occurred 4 hours later. The causes of the unfavorable results are to be sought in the two or three main effects of the protargol: (a) on the leukocytes, which already present, were greatly injured; (b) on the meningococci, which doubtless were also affected through direct bactericidal action from which toxic substances were set free; and (c) finally on removal of all restraint to

multiplication of surviving diplococci, since the immense numbers present in the lymph spaces as well as in the exudate indicate rapid growth.

A repetition of the experiment in which protargol was used was made in Experiment 8.

*Experiment 8. Treatment with Protargol.*—11.15 a.m., a *Macacus rhesus* received intraspinal inoculation of one agar slant culture, 18 hours old. 3.15 p.m., slightly ill; on perch. Lumbar puncture yielded cloudy fluid; culture positive. Films showed few leukocytes and many free meningococci. Injected 2 cc. of 0.2 per cent protargol suspension. 4.00 p.m., animal prostrate and spastic. 5.00 p.m., refused to rise. Died 5.00 a.m. next day. Survived 18 hours.

*Autopsy.*—No obvious visceral lesions outside the central nervous system. Turbid fluid in meninges, especially evident in sulci of cerebral convolutions. The general gray color of the cortex was about normal, but two symmetrical rectangular areas measuring 2 by 4 cm. were deeply congested and contained petechial hemorrhagic spots. They lay anterior and posterior to the fissure of Sylvius and extended to the midparietal sulcus comprising the ascending and inferior marginal convolutions. A few separate hemorrhages existed in the first temporal lobe, and the congestion and hemorrhages appeared to be confined to the gray matter. Cultures from the heart's blood and from several levels of the spinal cord and different surfaces of the brain were positive for the meningococcus. Film preparations showed many diplococci, few if any leukocytes, and little phagocytosis. Impression films prepared from the congested area of the brain contain scattered meningococci, few leukocytes, and almost no phagocytosis.

Sections from the brain show edema of and many leukocytes and red corpuscles within the meninges. The exudate contains many diplococci, both extra- and intracellular. In places the diplococci extend into the superficial layer of the cortex and slightly into the perivascular lymph spaces. These are far less numerous than in the 8 hour specimens. The affected cortex is congested and is the seat of many hemorrhages of varying size. The escape of blood is partly into the tissues and partly into the perivascular lymphatics. There is no obvious special relation between the hemorrhages and the diplococci.

A control for this monkey which received an identical quantity of an 18 hour culture was made ill, but recovered without treatment and following the spontaneous phagocytosis of the meningococci.

The differences in general disadvantageous to the protargol arose also in instances in which neither the untreated control nor the treated monkeys succumbed. In these instances cultures 24 hours old were employed. The symptoms set in more slowly than when 18 hour cultures were used and became less severe, abating wholly later.

But the monkeys given protargol suffered intensification of the symptoms almost immediately after the injection and recovered much more slowly than the untreated animals. In conformity with this fact it was found that the meningococci survived longer in the cerebrospinal fluid in the treated animals than in the control. A single experiment is given to bring out the distinction mentioned.

*Experiment 9. Control.*—8.50 a.m., a *Macacus rhesus* received an intraspinal inoculation of 1½ agar slant cultures of meningococci. 2.50 p.m., slightly ill. Turbid lumbar puncture fluid gave growth. The films showed numerous leukocytes, few meningococci, and little phagocytosis. Blood cultures were negative. The symptoms progressed slowly and never became severe. 24 hours after inoculation, the animal was still ill, but improving. Lumbar puncture yielded turbid fluid, which gave negative cultures. Film preparation of the fluid showed numerous leukocytes and few meningococci all within phagocytes. Within another 24 hours the animal had recovered.

*Experiment 10. Treated.*—8.30 a.m., a *Macacus rhesus* received intraspinally 1½ agar slant cultures. 12.30 p.m., ill. The turbid lumbar puncture fluid gave positive cultures. The film showed many leukocytes, few meningococci, and slight phagocytosis. Injected 1 cc. of 0.2 per cent protargol suspension. 1.30 p.m., very ill. 5.00 p.m., condition unchanged; blood culture positive. Turbid lumbar puncture fluid gave positive cultures. Film showed many leukocytes, of which some contained meningococci; many extracellular meningococci also. 10.00 p.m., animal lying on bottom of cage; aroused with difficulty. 24 hours after inoculation, improving. Turbid lumbar puncture of fluid still showed intra- and extracellular diplococci. No growth was obtained in culture. At the expiration of another 24 hours, the animal had still further improved, but several days elapsed before recovery was complete.

### *Effects of Lysol.*

The fact has already been mentioned that lysol has been employed in the treatment of epidemic meningitis with at first, as was supposed, beneficial results. Although a wider employment led very soon to its abandonment, yet the outcome of the studies of protargol suggested determining also the manner in which lysol acts. For this purpose small guinea pigs were employed. Two sets of tests were made: one in which the inoculated animals were allowed to succumb, the other in which they were chloroformed at the expiration of 6 hours, in order to ascertain just what changes were taking place in the peritoneal cavity. The lysol was employed in

1 per cent solution, which was the strength used in human cases of meningitis. The injection was made immediately following the injection of the culture, both being given into the peritoneal cavity (Table IV).

TABLE IV.

*Experiment 11. Therapeutic Test of Lysol.*

Weight of guinea pig.	Quantity of culture inoculated.	Treatment.	Result.
gm.			
100	1 m.l.d.	None (control).	Died in 10½ hrs.
100	1 "	1 cc. lysol (control).	Survived.
100	1 "	0.5 cc. lysol.	Died in 7 hrs.
100	1 "	0.5 cc. lysol + 0.5 cc. antiserum.	" "32 "
100	1 "	0.25 cc. lysol + 0.5 cc. antiserum.	" "20 "
100	½ "	0.5 cc. lysol.	" "12 "

*Guinea Pig 1. Control.*—Peritoneal fluid clear. 0.05 cc. plated gave innumerable colonies of meningococci, as did the same quantity of heart's blood. Films of peritoneal fluid showed enormous numbers of meningococci, but no cells. Films from the omentum showed extremely few leukocytes containing meningococci.

*Guinea Pig 2.*—Considerable amount of clear peritoneal fluid. 0.05 cc. plated gave innumerable colonies. Same amount from heart's blood gave 250 colonies. Films of the peritoneal fluid showed large numbers of diplococci, well preserved but agglutinated into small clumps. No leukocytes present. Films of the omentum showed a considerable number of leukocytes, many containing diplococci and many extracellular diplococci agglutinated.

*Guinea Pig 3.*—Very small amount of turbid peritoneal fluid. 0.05 cc. plated gave innumerable colonies. Same quantity of heart's blood gave 200 colonies of meningococci. Films of peritoneal fluid showed numerous leukocytes filled with diplococci, and large numbers of diplococci arranged in small clumps remaining extracellular.

*Guinea Pig 4.*—Small amount of turbid peritoneal fluid. 0.05 cc. plated gave 1,000 colonies. Same quantity of heart's blood gave no colonies. Films of peritoneal fluid showed numerous leukocytes, some staining badly, many containing diplococci, and a considerable number of small clumps of extracellular diplococci, some partly disintegrated. Films of the omentum showed more phagocytosis and a smaller number of extracellular diplococci.

*Guinea Pig 5.*—A considerable amount of turbid peritoneal fluid. 0.05 cc. plated gave innumerable colonies, as did the heart's blood. Films of the peri-

toneal fluid showed many leukocytes and a small amount of phagocytosis, besides extracellular clumps of diplococci. Films of the omentum showed more phagocytosis than the peritoneal fluid and a larger number of leukocytes, besides extracellular diplococci.

*Guinea Pig 6.*—A considerable amount of turbid peritoneal fluid. 0.05 cc. plated gave innumerable colonies, as did the heart's blood. Films of the peritoneal fluid showed numerous leukocytes, a small amount of phagocytosis, and numerous clumped extracellular diplococci. Films of the omentum showed numerous leukocytes, slight phagocytosis, and extracellular diplococci occurring in clumps.

### *Mechanism of the Action of Lysol.*

The mode of the action of lysol was studied by inoculating three young guinea pigs with one minimal lethal dose of culture and treating them immediately with 1 per cent lysol or 1 per cent lysol plus antiserum, as indicated in Table V.

TABLE V.  
*Experiment 12.*

Weight of guinea pig.	Quantity of culture inoculated.	Treatment immediately after inoculation.	Condition after 6 hrs.
gm.			
100	1 m.l.d.	0.5 cc. lysol + 0.5 cc. water.	Dead.
100	1 "	0.25 " " + 0.75 " "	Very sick.
100	1 "	0.25 " " + 0.25 " " + 0.5 cc. antiserum.	Prostrate.

The two guinea pigs which still survived were chloroformed and autopsies performed at once.

*Guinea Pig 1.*—Peritoneal fluid clear. 0.05 cc. plated gave innumerable colonies of meningococci. Same quantity of heart's blood gave 100 colonies. Films of the peritoneal fluid showed many meningococci and few leukocytes, there being almost no phagocytosis, while the leukocytes stained feebly. The meningococci were chiefly aggregated into clumps of 10 or 20 individuals. They stained as a rule diffusely and not sharply. The omentum showed many more leukocytes, almost all containing meningococci singly and in small clumps. The phagocytes stained feebly.

*Guinea Pig 2.*—Peritoneal fluid clear. 0.05 cc. gave innumerable colonies of meningococci. Same quantity of heart's blood gave about 100 colonies. Films of peritoneal fluid and omentum showed a condition similar to No. 1.

*Guinea Pig 3.*—Peritoneal fluid turbid, gave 150 colonies of meningococci and the same quantity of heart's blood gave three colonies. Films from the peritoneal cavity showed no diplococci whatever, either free or in leukocytes, of which a good number in excellent preservation were present. Films from the omentum showed large numbers of well preserved leukocytes, of which a few contained meningococci, either sharply stained or disintegrating; extracellular diplococci were practically absent.

It may in brief be stated that the experiments with guinea pigs indicate that lysol is antileukotactic to an even greater degree than is protargol. Moreover, the antiserum is capable in this case of overcoming to even a further extent the negative chemotactic and antiphagocytic action of the chemical. Obviously the combined toxic power of the chemical and the disintegrating effect upon the meningococci, notwithstanding the action of the serum, bring about a fatal issue.

#### DISCUSSION.

The results of the tests with protargol and with lysol are consistent. The chemicals have shown themselves not to be curative, but rather to be injurious in experimental meningococcic infection in guinea pigs and monkeys. Far from exhibiting power to convert a fatal into a non-fatal issue, they have shown rather a reverse tendency. Even when, as in certain tests on monkeys, recovery occurred after employing protargol, the symptoms of the treated animals were intensified as compared with the untreated control animals.

The explanation of the unfavorable effect of the chemicals is supplied by the study of the processes occurring in the peritoneum of guinea pigs and the subarachnoid space of monkeys. The key to recovery from infection with the meningococcus is furnished by the phenomenon of phagocytosis. Whatever means promote phagocytosis under conditions in which the leukocytes remain potent, facilitate recovery; whatever means lead to the reverse effect interfere with recovery. The chemicals have been shown to be antileukotactic, antiphagocytic, and indeed to be cell poisons of considerable power. Hence when they are brought into relation with the seat of infection, they prevent emigration of leukocytes on an adequate scale, and they reduce phagocytosis by such leukocytes as have entered the serous cavities.



Moreover, they also injure and bring about degeneration of the leukocytes themselves.

The resultant of this set of injurious activities is to open the way for a free invasion of the blood by the meningococci and for almost unrestrained multiplication in the serous cavities.

The chemicals do exert a direct bactericidal action upon such meningococci as come under their influence under conditions of suitable concentration, but not all the meningococci are thus destroyed. Those which survive multiply almost without hindrance; while the absence of detoxicating power on the part of the chemical permits the disintegration products of the destroyed meningococci to exert their poisonous effect upon the animal organism.

The manner of action of the chemicals is precisely the reverse of the antiserum. The latter acts leukotactically and brings into the infected serous cavities a far larger number of leukocytes than would otherwise appear there in the same period of time. Moreover, by preparing the meningococci for phagocytosis by supplying opsonin and by facilitating that process by agglutinating the diplococci, the serum greatly promotes the englobing of the microorganisms. Finally, through the possession of antitoxic power it neutralizes whatever endotoxin may be liberated by the disintegrating diplococci.

It is, therefore, not remarkable to find, as the experiments have shown, that the antiserum is capable of overcoming part of the defects of the chemicals. Because of its leukotactic and phagocytic properties, the antiserum removes some of the damage which the chemicals produce on account of their antagonism to those essential phenomena. But combinations of the chemical and the antiserum have proven less effective in combating experimental meningococcic infection than the antiserum alone, from which the deduction may be made that whatever benefit may attach to effective direct bactericidal action by the chemicals, irrespective of the type of meningococcus causing the infection, is lost because of the dangers arising from their antiphagocytic effects and lack of detoxicating properties.

Doubtless the introduction of the chemicals into the subarachnoid space in man has been made without the uniformly serious consequences observed in our experiments on animals. The reason for this

discrepancy is perhaps obvious. The relative dosage, considering comparative size and weight, was far less in man than in the animals. Hence it may be assumed that the injurious effects, if any were produced, were masked. To assume, on the other hand, that because no evidence of untoward action was detected in the treatment with protargol, and five out of eight cases of epidemic meningitis treated with that chemical recovered, the drug has curative properties, is to disregard previous experience with simple lumbar puncture, with lysol, and with other methods of treatment which for a time and because of the variable severity of cases of epidemic meningitis seemed to offer encouragement. It is not probable that such active protoplasmic poisons as protargol and lysol can be employed with impunity for direct introduction into the closed cavity of serous membranes, the seat of the meningococcic infection.

#### SUMMARY.

Claims of efficiency have been made at two widely separated periods for the chemical treatment of epidemic meningitis, in the first instance for lysol and in the second for protargol. The use of lysol was long since abandoned; the recommendation for protargol is based on a single series of cases, small in number. Because of the variable severity of epidemics of meningitis, small reliance can be placed on results of treatment limited in extent to small numbers of cases and to one locality. A more uniform and accurate measure of the value of a method of treatment is provided by animals infected experimentally with pathogenic cultures of meningococci.

Young guinea pigs respond in a definite manner to intraperitoneal inoculation of virulent meningococci. Neither protargol nor lysol proved to have any curative action on the experimental infection thus produced in these animals.

Monkeys respond in a characteristic manner to the inoculation of virulent cultures into the subarachnoid space. Protargol displayed no curative action on the experimental infection thus produced in these animals.

On the contrary, both lysol and protargol exert antileukotactic and antiphagocytic effects, and are also potent protoplasmic poisons,

and the leukocytes with which they come in contact are injured and made to degenerate. According to the extent to which these harmful properties are exerted, the chemicals promote the advance rather than restrain the progress of meningococcic infection.

Recovery from meningococcic infection in man and animals is accomplished chiefly through the process of phagocytosis. The specific antiserum acts curatively by increasing the emigration of leukocytes, by promoting phagocytosis directly, and by agglutinating the meningococci, and also by neutralizing endotoxin. Any means which interfere with and reduce these essential processes retard or prevent recovery. Both lysol and protargol interfere with and diminish the emigration of leukocytes and the phagocytosis of meningococci, and neither possesses antitoxic power.

The mixture of antiserum with lysol and with protargol reduces to a certain extent the antileukotactic and antiphagocytic effect of the chemicals; but this action is insufficient to set aside wholly the injurious effects which they produce.

It follows, therefore, that whatever theoretical advantages might accrue from a bactericidal activity exerted by these chemicals independently of the type of meningococcus causing epidemic meningitis, is more than offset by the harmful effects which they cause.

Hence specific antiserum seems to provide the logical therapeutic agent with which to combat epidemic meningitis, since it is itself innocuous and promotes those processes essential to recovery from the disease. The problem up to the present has been that of producing an antiserum which represents the several types of the meningococcus, and this problem is now in a fair way to being solved.<sup>1</sup>



# EXPERIMENTS ON THE DEVELOPMENT OF MALARIA PARASITES IN THREE AMERICAN SPECIES OF ANOPHELES.

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PLATES 98 TO 105.

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The early results of experiments to determine the susceptibility of *Anopheles punctipennis* to infection with the parasites of tertian malaria have been recorded in two recent articles by the writer (1915-1916). In these it was shown that this species is an efficient host of *Plasmodium vivax*, and in the second article a foot-note was appended stating that it had also proven to be a host of *Plasmodium falciparum*, the parasite of estivo-autumnal malaria.

In the present paper are given further details of this series of experiments in which the susceptibility of *Anopheles crucians*, as well as of *Anopheles punctipennis*, has been established. The information acquired upon the relative susceptibility of these two species together with *Anopheles quadrimaculatus* is also included.

A study of the literature applying to this subject has revealed the fact that an extremely small amount of experimental work has actually been done with the species of *Anopheles* occurring in the United States. The experiments with *Anopheles punctipennis* were contradictory and inconclusive, and, as stated in the previous articles, the impression had become current that it was not a factor in the transmission of malaria. The proof of its efficiency as a host of malaria parasites does not, of course, establish the degree of its importance in the transmission of malaria. This depends as well upon its food habits in relation to man, its prevalence, and distribution.

In regard to *A. crucians*, nothing has been found in the early literature which establishes with certainty the proof of its susceptibility, although it has been included among the efficient hosts by several authors, notably Howard, Dyar, and Knab (1912), and Knab (1913). They refer to the experiments reported by Beyer, Pothier, Couret, and Lemann (1902), as Dupree (1905) undoubtedly does also in accrediting the proof to Couret and Beyer. The statements are evidently based upon a misinterpretation of the report.

The results of the experiments by Beyer and his associates may be summarized as follows: With *A. crucians*, no infections were obtained with tertian parasites in two specimens or with quartan parasites in three specimens, while none were tested with estivo-autumnal malaria. With *A. quadrimaculatus*, one specimen of three became infected with tertian malaria and two specimens of five with quartan. The five fed on blood containing estivo-autumnal parasites were negative. Their incrimination of *A. crucians* as a host of estivo-autumnal malaria was based on epidemiologic considerations.

Mitchell (1907) made the statement that Dupree had shown her the parasites in the salivary glands of three species (*A. quadrimaculatus*, *A. crucians*, and *A. punctipennis*). Dupree himself, however, made no reference to his having obtained infected specimens of *quadrimaculatus* or *crucians*.

Recently Mitzmain (1916) obtained negative results with 219 *A. punctipennis* dissected from 3 to 38 days after multiple bites on individuals whose blood contained varying numbers of estivo-autumnal crescents. One specimen of three *A. crucians* fed on one of the gamete carriers showed an infection.

The susceptibility of *A. quadrimaculatus* was established by the work of Thayer (1900), who obtained positive results with this species for both estivo-autumnal and tertian malaria—one infected specimen with the former and two with the latter). Woldert (1901) conducted nine experiments with *A. quadrimaculatus*. The first experiment with tertian malaria failed. Fourteen specimens dissected in the following six experiments with tertian were all negative. In the last two, with estivo-autumnal malaria, two positive specimens were found among the seven dissected. Berkeley (1901) succeeded after repeated experiments in inoculating *A. quadrimaculatus* with the tertian parasite. Hirshberg (1904) obtained eight infected *A. quadrimaculatus* in a series of forty-eight fed on five estivo-autumnal gamete carriers. Beyer, Pothier, Couret, and Lemann reported infections in this species after the ingestion of the parasites of tertian and quartan malaria.

A fourth species of *Anopheles* (*A. pseudopunctipennis*), reported from the Southern United States, was found positive for the parasite of estivo-autumnal malaria in experiments by Darling (1910) in the Panama Canal Zone. Out of thirty-one specimens dissected, four were found to be infected. From the small number infected under the most favorable artificial conditions, and because of the fact that relatively few of this species are taken in dwellings, Darling concludes that *pseudopunctipennis* is only slightly concerned in the transmission of malaria.

The importance of the accurate determination of the exact relation of each species of *Anopheles* to malaria transmission has been emphasized by the results of a number of noteworthy investigations, which have shown that the greatest variation in susceptibility exists among the different species of this genus. Some species are entirely immune, while certain species manifest a difference in susceptibility to the different species of malaria plasmodia. Walker and Barber (1914) have given an excellent discussion of these phases of the subject in the introduction to their paper on "Malaria in the Philippine Islands."

#### EXPERIMENTAL.

The experiments reported below were conducted in New Orleans during the months of November and December, 1915, and January, 1916. The adult mosquitoes of the two species, *Anopheles quadrimaculatus* and *Anopheles punctipennis* were bred from larval material supplied by Mr. D. L. Van Dine, of the Bureau of Entomology, from Mound, La. Since no bred material of *Anopheles crucians* was available, specimens collected in the open were used. A flight of this species into the city occurred at this time and upon examination it was found that only a very small proportion of them had had blood meals. The specimens used for infecting purposes were selected from the ones in which no distention of the abdomen with blood or developed ova could be detected.

The experimental mosquitoes were given but one meal of infected blood. They were fed individually, not in lots as is usually done, so that the fact of their having fed was thus made certain in each case. Before the blood meal and afterwards, the diet consisted of raisins and water. Between the times of the blood meal and dissection, each specimen was kept in a separate container.

In Table I is given a part of the data obtained from the examinations of females of *Anopheles punctipennis* after they had fed on estivo-autumnal gamete carriers. The examinations were made at intervals ranging from 7 to 46 days. In all, twenty-two specimens were fed on two patients. Two specimens were not examined. On one of the patients four feedings were made on different days. Case 514 showed a medium number of gametes in the blood, but since no other species besides *punctipennis* were fed on this case, a satisfactory explanation of the negative results cannot be made.

TABLE I.

*Results of Experiments with Anopheles punctipennis and Estivo-Autumnal Parasites.*

Specimen No.	Date of feeding.	Date of dissection.	Interval.	Result of examination.		Case No.
				Midgut.	Salivary glands.	
	1915	1915	days			
602.1	Nov. 13	Dec. 30	47	—	+	511
602.2	" 13	" 15	32	—	—	511
617.1	" 13	Nov. 25	12	—	Not examined.	511
617.2	" 13	—				511
617.3	" 13	Dec. 6	23	—	—	511
618.1	" 13	" 29	46	—	—	511
618.2	" 13	" 27	44	—	—	511
618.3	" 13	" 27	44	—	—	511
612.6	" 22	" 21	29	—	—	511.5
626.1	" 22	" 21	29	+	+	511.5
617.4	" 23	" 14	21	+	+	511.6
618.5	" 23	Nov. 30	7	—	—	511.6
620.1	" 23	Dec. 15	22	+	—	511.6
		1916				
601.1	Dec. 23	Jan. 17	25	—	—	514
601.2	" 23	" 14	22	—	—	514
601.3	" 23	—				514
601.4	" 23	" 17	25	—	—	514
604.1	" 23	" 15	23	—	—	514
606.1	" 23	" 15	23	—	—	514
607.1	" 23	" 14	22	—	—	514
607.2	" 23	" 14	22	—	—	514
610.1	" 23	" 17	25	—	—	514

Total No. dissected..... 20

" " infected..... 4

Per cent " ..... 20

" " " of those fed on Case 511 only..... 33

In Specimen 602.1, the salivary glands contained only a very few sporozoites. In No. 626.1, one oocyst and one empty capsule were found upon the stomach, and the salivary glands contained large numbers of sporozoites in all the lobes of both sets. In No. 617.4 (Figs. 7, 8, and 14), one oocyst and one empty capsule were present on the stomach. The oocyst showed well developed sporoblasts. The salivary glands of this specimen were heavily infected with



sporozoites. In No. 620.1, eight oocysts were counted on the stomach, five of which were placed toward the anterior end of the enlargement of the midgut. Two of the oocysts were in the last stage of development before the release of the sporozoites, and four were large but without visible sporoblasts. The measurement of the latter gave: 47 by 48, 31 by 37, 35 by 40, and 48 by 55 microns. The other two were very small—not over 20 microns in diameter.

In Table II are shown the results of the examination of females of *Anopheles punctipennis* after feeding on tertian gamete carriers. Further details of this series have been given in a previous article (King, 1916).

TABLE II.

*Results of Experiments with Anopheles punctipennis and Tertian Parasites.*

Specimen No.	Date of feeding.	Date of dissection.	Interval.	Result of examination.		Case No.
				Midgut.	Salivary glands.	
	1915	1915	days			
612.1*	Nov. 6	Nov. 24	18	+	—	509
612.2	" 6	" 15	9	+	Not examined.	509
612.3	" 6	Dec. 1	25	—	—	509
614.1†	" 6	Nov. 26	20	+	+	509
614.2	" 6	" 13	7	+	Not examined.	509
614.3	" 6	" 13	7	+	" "	509
612.5‡	" 12	Dec. 2	20	+	+	510

Total No. dissected.....	7
" " infected.....	6
Per cent " .....	85

\* Figs. 2, 3, and 4. † Figs. 9 and 11. ‡ Fig. 10.

Table III shows the results obtained with specimens of *Anopheles crucians* fed upon an estivo-autumnal gamete carrier. A total of thirty-five females were fed during a period of 5 days, but this species lived poorly in captivity, and sixteen which died soon after the blood meal were unsuitable for examination. The fact that they were "wild" mosquitoes and not bred, as were the other two

species, may account for this high mortality. Seven other specimens which were used in another experiment could not be included in the tabulation.

TABLE III.

*Results of Experiments with Anopheles crucians and Estivo-Autumnal Parasites.*

Specimen No.	Date of feeding.	Date of dissection.	Interval.	Result of examination.		Case No.
				Midgut.	Salivary glands.	
	1915	1915	days			
703.1	Nov. 23	Dec. 7	14	—	—	511.6
703.2	" 23	" 16	23	—	—	511.6
703.4	" 23	" 3	10	—	Not examined.	511.6
703.6	" 23	" 13	20	+	—	511.6
703.8	" 23	" 16	23	—	+	511.6
703.9	" 23	" 20	27	—	+	511.6
703.12	" 23	" 9	16	+	Not examined.	511.6
705.8	" 24	" 24	30	—	+	511.7
709.1	" 27	" 27	30	+	+	511.9
710.2	" 27	" 21	24	+	—	511.9
710.3	" 27	" 26	29	+	+	511.9
710.4	" 27	" 27	30	+	—	511.9

Total No. dissected..... 12

" " infected..... 9

Per cent " ..... 75

Specimen 703.6 (Figs. 1, 5, and 6), had a very large number of oocysts on the stomach; the number was estimated at 75 after 39 had been counted. These ranged in size from 24 to 50 microns in diameter, the majority probably between 40 and 50. No. 703.8 had a medium infection of the center lobes of the salivary glands (compare Fig. 12). A heavy infection of all the lobes of the glands existed in No. 703.9. Five oocysts were present on the stomach of No. 703.12. Two of these measured 34 by 37 microns. In No. 705.8 a small number of sporozoites were found in the salivary glands. In No. 709.1, one large oocyst, 50 by 60 microns, was present on the stomach. The condition of the salivary glands was such that the presence of sporozoites could not be definitely ascertained. One oocyst measuring 45 microns in diameter was found on the stomach

of No. 710.2. In No. 710.3, one oocyst was present on the stomach and the glands seemed to contain sporozoites, but their condition made the diagnosis uncertain. In No. 710.4, one oocyst was seen on the stomach.

Table IV shows the infections occurring in specimens of *Anopheles quadrimaculatus* with tertian parasites, and Table V with estivo-autumnal parasites.

TABLE IV.

*Results of Experiments with Anopheles quadrimaculatus and Tertian Parasites.*

Specimen No.	Date of feeding.	Date of dissection.	Interval.	Result of examination.		Case No.
				Midgut.	Salivary glands.	
	1915	1915	days			
349.2	Nov. 2	Nov. 11	9	—	Not examined.	507
351.3	" 2	" 6	4	—	" "	507
352.7	" 2	" 10	8	—	" "	507
352.8	" 2	" 9	7	+	" "	507
352.9	" 2	" 12	10	+	" "	507
365.1*	" 6	Dec. 7	31	+	+	509
365.2	" 6	—				509
372.1	" 6	—				
373.1	" 6	Nov. 16	10	+	—	509
373.2	" 6	" 19	13	+	Not examined.	509
373.3	" 6	—				
347.6	" 12	Dec. 9	27	—	+	510
365.5†	" 12	" 9	27	—	+	510
365.6	" 12	" 13	31	—	—	510
371.1	" 12	—				
373.4	" 12	—				
373.5‡	" 12	Nov. 30	18	—	+	510
374.1	" 12	—				

Total No. dissected.....	12
" " infected.....	8
Per cent " .....	66

\* Figs. 16 and 17. † Fig. 13. ‡ Fig. 15.

TABLE V.

*Results of Experiments with Anopheles quadrimaculatus and Estivo-Autumnal Parasites.*

Specimen No.	Date of feeding.	Date of dissection.	Interval.	Result of examination.		Case No.
				Midgut.	Salivary glands.	
	1915	1915	days			
349.1	Nov. 2	Nov. 13	11	—	Not examined.	506
354.1	" 2	" 10	8	—	" "	506
358.1	" 2	" 15	13	—	" "	506
358.2	" 2	" 15	13	—	" "	506
359.1	" 2	—				506
359.2	" 2	" 8	6	—	" "	506
359.3	" 2	" 8	6	—	" "	506
347.7	" 13	Dec. 6	23	—	—	511
350.1	" 13	" 1	18	—	—	511
350.2	" 13	—				
350.3	" 13	" 29	46	—	—	511
350.4	" 13	" 18	35	—	+	511
365.7	" 13	" 28	45	+	—	511
371.2	" 13	Nov. 20	7	—	Not examined.	511
380.1	" 22	—			—	511.5
386.1	" 23	Dec. 14	21	—	—	511.6
390.1	" 23	" 14	21	+	—	511.6
381.1	" 24	" 21	27	—	—	511.7
803.1	" 24	" 15	21	—	—	511.7
381.2	" 26	—				511.8
394.1	" 27	" 22	25	—	—	511.9
396.1	" 29	" 13	14	—	—	511.10
396.2	" 29	" 22	23	—	—	511.10

Total No. dissected..... 19

" " infected..... 3

Per cent " ..... 15

" " " of those fed on Case 511..... 23

Since no infections resulted from Case 506, the percentage from Case 511 only is shown.

The total number of mosquitoes of the three species examined is too small to permit of very satisfactory comparisons, but the indications of relative susceptibility as shown in Tables VI and VII are of interest. In these only those specimens which were fed on the same gamete carriers have been included. Since the females of

*Anopheles crucians* were not bred, while those of the other two species were, the results are perhaps not strictly comparable.

TABLE VI.  
*Comparative Results with Plasmodium vivax.*

	<i>A. punctipennis.</i>	<i>A. quadrimaculatus.</i>
Total No. dissected.....	7	7
" " infected.....	6	6
Per cent ".....	85	85

TABLE VII.  
*Comparative Results with Plasmodium falciparum.*

	<i>A. punctipennis.</i>	<i>A. quadrimaculatus.</i>	<i>A. crucians.</i>
Total No. dissected.....	12	13	12
" " infected.....	4	3	9
Per cent ".....	33	23	75

In Table VIII are shown the proportion of gametes to leukocytes in the blood upon which the mosquitoes fed. Case 511 was employed for feeding purposes on several different days extending over a period of 2 weeks. As may be seen from the earlier counts, the numbers of gametes were extremely high.

TABLE VIII.  
*Comparative Counts of Leukocytes and Gametes Made from Stained Blood Smears Taken at the Time of the Feeding of the Mosquitoes.*

Case No.	Species of <i>Plasmodium.</i>	No. of leukocytes counted.	Gametes.	Gametes per 100 leukocytes.
509	<i>P. vivax.</i>	131	19	14
511	" <i>falciparum.</i>	57	300	526
511.5	" "	100	143	143
511.6	" "	200	187	93
511.7	" "	125	171	136
511.8	" "	350	106	30
511.9	" "	350	128	36
511.10	" "	300	56	18
514	" "	281	20	7

The mosquitoes used in the experiments were kept in a darkened, screened box in the laboratory. Temperature and humidity records for the entire period were kept by means of Friez recording instruments placed beside the box. The temperature of this room was usually higher and fluctuated less than the outdoor temperature, and during part of the time the room was artificially heated during the day. The temperature in the laboratory rarely fell below 60°F. On Dec. 28, however, the minimum was 51°, and on the 29th, 49°.

TABLE IX.

*Weekly Mean Temperature and Per Cent Humidity (Relative) from November 1, 1915, to January 16, 1916.*

Week.	Mean temperature.	Average relative humidity.
	°F.	per cent
Nov. 1-7	76.7	60.3
" 8-14	79.7	65.5
" 15-21	71.6	48.7
" 22-28	73.5	57.6
" 29-Dec. 5	68.5	48.8
Dec. 6-12	72	64.0
" 13-19	68.7	62.0
" 20-26	65.4	54.2
" 27-Jan. 2	64.7	72.2
Jan. 3-9	75	64.4
" 10-16	69.1	51.6

An explanation of the long developmental period of the parasites, as exhibited in these experiments, is undoubtedly found in the temperature conditions prevailing during the time. The length of the sexual cycle is usually given as from 9 to 12 days, but the exact relation of temperature to the period of development has not been carefully ascertained.

## SUMMARY.

Since a knowledge of the susceptibility of any species of *Anopheles* to infection with malaria parasites is of great importance in determining its part in the transmission of malaria, the experiments reported here were undertaken, and included the three most prevalent species of this genus occurring in the United States. As a result of these experiments *Anopheles punctipennis* is shown to be an efficient

host of the organisms of tertian and estivo-autumnal malaria, *Anopheles crucians* of estivo-autumnal malaria, at least, and information has been obtained upon the relative susceptibility of these two species and *Anopheles quadrimaculatus*. The latter species has been known to be an efficient host since Thayer's experiments in 1900, and has been considered to be the principal species concerned in the transmission of malaria in the United States.

With *Anopheles punctipennis*, developmental forms of the exogenous or sporogenic cycle of *Plasmodium vivax* were demonstrated in six (85 per cent) of the seven mosquitoes dissected, and the development of *Plasmodium falciparum*, in four (20 per cent) of twenty specimens. These four infections, however, occurred in a series of thirteen specimens fed on one person, so that the percentage was actually 33.

With *Anopheles crucians*, oocysts or sporozoites or both oocysts and sporozoites of *Plasmodium falciparum* were found in nine (75 per cent) of the twelve specimens dissected. No tests were made with this species and *Plasmodium vivax*.

*Anopheles quadrimaculatus* was employed as a control species in the experiments and became infected in the following ratio: eight (66 per cent) of twelve specimens with *Plasmodium vivax*, and three (15 per cent) of nineteen specimens with *Plasmodium falciparum*.

In determining the relative susceptibility of the three species only those individuals which had fed upon the same gamete carriers are considered. The number of mosquitoes from which the percentages are computed is too small to make the results entirely conclusive, but the indications are that *Anopheles punctipennis* and *Anopheles quadrimaculatus* are equally susceptible to infection with *Plasmodium vivax*, 85 per cent of each species under the same conditions being positive. With *Plasmodium falciparum*, *Anopheles crucians* showed the highest percentage of infection (75 per cent), *Anopheles punctipennis* second (33 per cent), and *Anopheles quadrimaculatus* third (23 per cent).

The writer desires to acknowledge the cooperation and advice received from Dr. C. C. Bass and Dr. F. M. Johns, of the Laboratories of Clinical Medicine of the School of Medicine of Tulane University.

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EXPLANATION OF PLATES.<sup>1</sup>

## PLATE 98.

FIG. 1. Oocysts of *Plasmodium falciparum* in *Anopheles crucians* (No. 703.6).

FIG. 2. Oocysts of *Plasmodium vivax* in *Anopheles punctipennis* (No. 612.1).

## PLATE 99.

FIG. 3. Two oocysts of *Plasmodium vivax* in *Anopheles punctipennis* (No. 612.1).

FIG. 4. Five oocysts of *Plasmodium vivax* in *Anopheles punctipennis* (No. 612.1).

## PLATE 100.

FIG. 5. Eight oocysts of *Plasmodium falciparum* in *Anopheles crucians* (No. 703.6).

FIG. 6. Ten oocysts of *Plasmodium falciparum* in *Anopheles crucians* (No. 703.6).

## PLATE 101.

FIG. 7. An oocyst of *Plasmodium falciparum* in *Anopheles punctipennis* (No. 617.4), showing formation of sporoblasts.

FIG. 8. Same as Fig. 7, more highly magnified.

## PLATE 102.

FIG. 9. An oocyst of *Plasmodium vivax* in *Anopheles punctipennis* (No. 614.1). This body contained active sporozoites and was at the point of rupturing.

FIG. 10. The empty capsule of an oocyst of *Plasmodium vivax* in *Anopheles punctipennis* (No. 612.5), after the escape of the sporozoites.

FIG. 11. Two empty capsules of oocysts of *Plasmodium vivax* in *Anopheles punctipennis* (No. 614.1).

## PLATE 103.

FIG. 12. A normal salivary gland of *Anopheles crucians*, showing the relation of the three lobes.

FIG. 13. A mass of sporozoites of *Plasmodium vivax* in *Anopheles quadrimaculatus* (No. 365.5). These were expelled from the salivary glands by the pressure of the cover glass.

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<sup>1</sup> The author is greatly indebted to Dr. F. M. Johns for aid in the preparation of the microphotographs of infected mosquitoes, and to Dr. W. S. Thayer of Baltimore for confirmation of his interpretation of several of the preparations.

## PLATE 104.

FIG. 14. Sporozoites of *Plasmodium falciparum* in glands of *Anopheles punctipennis* (No. 617.4). (Although a very poor illustration it has been included since it is the only microphotograph obtained of sporozoites in this species of mosquito.)

FIG. 15. Sporozoites of *Plasmodium vivax* in *Anopheles quadrimaculatus* (No. 373.5).

## PLATE 105.

FIG. 16. Sporozoites of *Plasmodium vivax* in *Anopheles quadrimaculatus* (No. 365.1). This illustrates the typical clumping in one of the gland cells.

FIG. 17. Same as Fig. 16, more highly magnified.

|

FIG. 1.

FIG. 2.  
(King: Development of Malaria Parasites.)



FIG. 3.

FIG. 4.

(King: Development of Malaria Parasites.)



FIG. 5.

FIG. 6.

(King. Development of Malaria Parasites.)





FIG. 7.

FIG. 8.

(King: Development of Malaria Parasites.)





FIG. 9.

FIG. 10.



FIG. 11.

(King: Development of Malaria Parasites.)



FIG. 12.

FIG. 13.

(King: Development of Malaria Parasites.)



|

FIG. 12.

|

FIG. 13.

(King: Development of Malaria Parasites.)





## INTESTINAL OBSTRUCTION.

### VI. A STUDY OF NON-COAGULABLE NITROGEN OF THE BLOOD.

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(Received for publication, March 3, 1916.)

This communication deals with analyses of the blood in intestinal obstruction, intestinal closed loops, and other acute intoxications. The tables give figures for non-coagulable nitrogen, urea nitrogen, and in some instances the total nitrogen partition in the blood. Our interest in this study of the blood was aroused by a communication of Tileston and Comfort (1), who in a large series of human cases reported three cases of intestinal obstruction with very high non-coagulable nitrogen. Since that time we have studied the blood of various experimental animals which were being observed in connection with other experimental work.

We found that most cases of intestinal obstruction, especially with signs of acute intoxication, showed a high non-coagulable blood nitrogen, and it seemed possible that this factor might be of value in diagnosis and especially prognosis of acute abdominal conditions. We are now convinced that this non-coagulable nitrogen determination is of value in various acute intoxications. If the reading is high, one may assume a dangerous grade of intoxication, but, on the contrary, one may not assume that a low reading gives evidence of slight intoxication, because a fatal outcome may be associated with a low reading. It is of considerable value to know that the non-coagulable nitrogen of the blood may show high readings in other conditions besides renal disease.

On the other hand, determinations of the blood urea alone are of somewhat less value in studying the retention products in the blood in these conditions. In our experimental animals the blood urea

has varied from less than 30 per cent to more than 80 per cent of the total non-coagulable nitrogen, and, while a high urea reading is the rule, the variations in the urea curve and the curves of the other non-coagulable nitrogenous substances are so great that the urea reading is a somewhat unreliable index of the extent to which non-coagulable nitrogenous substances have accumulated.

Our results are given below in the form of tables and some clinical and experimental data are given in addition, but it is not needful to give the entire experimental data at this time. Usually one experiment in each table is given in some detail as an example of the group.

### *Methods.*

Dogs were used in most experiments. A few cats were used, and one human case was included. All operations on animals were done under surgical anesthesia with the usual surgical technique.

The blood was taken in a glass syringe from the jugular vein and used for the various tests. When the blood was obtained at the time the animal was killed, it flowed from a cannula in the carotid. A few samples taken *in extremis* were obtained after anesthesia by opening the thorax and aspirating direct from the heart.

Non-coagulable nitrogen is determined as follows: With a volumetric pipette 10 cc. of blood are added to 65 cc. of distilled water, and 5 cc. of a 1 per cent sodium oxalate solution. The mixture is brought to a boil, gently rotating the flask while heating, and is then faintly acidified with acetic acid. A few crystals of sodium sulphate are added, the mixture is shaken, and, after the addition of 20 cc. of a 1 per cent solution of uranium acetate, is thoroughly shaken again and filtered. Nitrogen estimations by the Kjeldahl method are done in duplicate on 30 cc. samples of the clear filtrate,—each representing 3 cc. of blood,—and the non-coagulable nitrogen calculated for 100 cc. Creatinine and creatine are determined by the method of Folin (2) with a standard of creatinine zinc chloride. Urea is determined by the method of Marshall as modified by Van Slyke and Cullen (3). Uric acid is determined by the method of Benedict (4).

### EXPERIMENTAL OBSERVATIONS.

#### *Simple Obstruction, Recovery, and Second Operation.*

*Dog 15-12.*—Mongrel, female; weight 40 pounds.

Mar. 8. A simple obstruction was made in the middle of the small intestine, and a small piece of tape was fixed about the small intestine to occlude its lumen but not to injure the intestinal wall.

Mar. 9. Dog quiet. Temperature 38.5°C. Slight vomiting.

Mar. 10. Dog shows usual picture of obstruction with vomiting. Temperature 38°C. Weight 38 pounds.

Mar. 11. Condition the same. Intoxication not striking. Weight 36 pounds.

Mar. 12. Condition the same. Weight 34 pounds. Late in the day the dog showed intoxication and small pulse. Infusion of 1,000 cc. of Locke's solution given in the jugular. Blood taken before and after infusion. The infusion caused clinical improvement.

Mar. 13. Dog still vomiting; the intoxication is not severe. Temperature 38.5°C. Weight 35.5 pounds. The marked drop in non-coagulable nitrogen is a striking feature of this experiment, and is difficult to explain, although the diuresis and replacement of lost fluid may be important factors. 1 p.m. Ether anesthesia and laparotomy. The obstruction was removed easily, and the intestinal wall closed over by sutures. At the end of the operation intravenous infusion of 1,000 cc. of Locke's solution. Dog made good recovery from operation.

Mar. 14. Dog vomited a little and refused food.

Mar. 15. Vomiting continues, and animal eats a little. Temperature 38.7°C. Weight 34 pounds. Given 1,000 cc. of Locke's solution intravenously.

Mar. 16. Much improvement; dog takes food; no vomiting. Weight 35 pounds.

Mar. 17 and 18. Continued improvement.

Mar. 19. Improvement continues; dog has passed feces.

Apr. 8. Dog appears normal. Second operation to establish a drained loop of duodenum including about 8 inches of the first part of the jejunum; distal portion of loop pulled through a puncture wound in left rectus; duodenum closed below pancreatic duct and gastrojejunostomy done.

Apr. 9. Dog sick, vomiting. Pulse weak. 3 p.m. Severe intoxication; much chocolate colored vomitus. Ether anesthesia. Killed.

*Autopsy.*—Thorax, heart, and lungs normal; abdominal viscera are normal; peritoneum is clean; gastro-enterostomy is patent; stomach shows slight engorgement of mucosa. Duodenum between pylorus and point of section shows a swollen and congested mucosa. It contains a blood-stained fluid. Jejunum below gastro-enterostomy shows engorgement of its mucosa very like that noted after lethal injection of a toxic proteose. The ileum and colon show congestion of their mucosæ to a less extent. The loop contains chocolate-red, slimy material; the mucosa shows no ulceration, but is congested and deep red; there are some submucosa ecchymoses.

This experiment is of interest because of the recovery from the first obstruction. An obstruction was produced in the middle of the small intestine by means of a tape. On the 6th day the tape was removed at a second operation, and the dog returned to a normal

TABLE I.

*Dog 15-12. Simple Obstruction, Recovery, and Second Operation.*

Day after operation.	Time.	Non-coagulable nitrogen.*	Remarks. Mongrel, female; weight 40 lbs.
		mg.	
2	2.00	23	Vomited once. Pulse good. Temp. 38.5°C.
3	11.00	24	Dog vomiting. Condition good. 38 lbs.
4	4.00	68	Dog vomiting frequently. 36 lbs. Temp. 38°C.
5	11.00	119	Dog vomiting. Pulse good. Feces in cage. 34 lbs. Temp. 38°C.
5	2.50	131	Dog prostrated. Pulse tension low. Infusion of 1,000 cc. of Locke's solution.
5	3.20	117	Blood after infusion. Clinical improvement.
6	11.30	31	Dog improved. Vomiting less.
2nd operation.	1.00	42	Laparotomy and removal of obstruction. Infusion of 1,000 cc. of Locke's solution. Blood at end of infusion.
7	11.00	36	Vomits occasionally. Refuses food.
9	10.00	26	Dog improving. 35 lbs.
11	4.00	28	Dog improving rapidly. 35.5 lbs. Temp. 38.4°C.
12	11.00	31	Dog passed feces. Seems quite well.
32	—	—	Normal.
3rd operation.	3.00	—	Drained loop of jejunum. Long operation.
33	12.00	56	Dog much intoxicated and vomiting. Urea nitrogen 31 mg.
33	3.00	71	Animal acutely sick. Killed. Amino nitrogen 9.3 mg.

\* Non-coagulable nitrogen is given in terms of mg. per 100 cc. of blood.

condition. The third operation was performed, and about 18 inches of the lower duodenum and first portion of the jejunum were isolated as a drained loop opening in the left side. A gastro-enterostomy was performed. The animal showed grave intoxication with much vomiting and death in 24 hours. There was no peritonitis but some interference with the blood supply of the drained loop.

Table I shows the fluctuation in the blood non-coagulable nitrogen. There was a steady rise to a maximum of 131 mg. per 100 cc. of blood on the 5th day. The dog was given an infusion, and the following day the non-coagulable nitrogen had dropped to 31 mg. One is tempted to assume as a simple explanation that the replace-

ment of fluid and the consecutive diuresis sweep out these substances from the blood. This undoubtedly does occur, but the story is not quite as simple as this. For example, in other experiments with a grave intoxication a transfusion may not affect in the least the level of blood non-coagulable nitrogen, in spite of a striking diuresis. There is no evidence to point to lack of eliminative power of the kidneys as a factor.

TABLE II.  
*Simple Obstruction.*

Dog No.	Day after operation.	Non-coagulable nitrogen.*	Urea nitrogen.*	Weight.	Temp.	Remarks.
		mg.	mg.	lbs.	°C.	
15-20	6	48	—	15.3	38.5	No vomitus. Dog quiet.
	7	33	—	14.5	38.4	Condition the same.
	8	38	—	14.3	39.1	" " "
	9	40	19	13.8	38.5	Little vomitus, intoxication moderate. Killed.
15-26	1	77	—	—	—	Blood after operation.
	3	46	34	42.8	39.2	Vomiting and sick.
	4	60	35	41.3	39.2	Condition the same.
	5	56	40	40.8	39.3	" " "
	6	60	39	40.0	39.1	" " "
	7	94	58	39.0	39.6	Dog weak.
	8	79	42	—	—	Dog seems better. Killed. Slight chronic nephritis.
16-45	1	34	15	36.0	—	Blood before operation.
	3	52	28	34.0	39.7	Vomiting large amounts.
	4	59	21	33.0	39.3	Vomiting.
	6	90	43	—	—	Dog acutely sick. Killed.
16-56	1	—	22	22.0	—	
	4	—	22	19.0	38.0	Dog vomiting since operation.
	5	63	21	—	37.7	11 a.m. Animal in fair condition. Proteose injection. Blood before injection.
	5	81	27	19.5	—	11.10 a.m. Recovered from proteose injection in 6 hrs. Died in night. Blood after injection.

\* Non-coagulable and urea nitrogen are given in terms of mg. per 100 cc. of blood.

*Simple Obstruction for 5 Days. Toxic Death.*

*Dog 16-45.*—Shepherd, mongrel, female; weight 36 pounds.

Nov. 10. Simple obstruction in the middle of the small intestine by means of complete section and an inversion of cut ends.

Nov. 11 and 12. Dog is vomiting, and there is a steady loss in weight.

Nov. 13. Much thin yellow vomitus. Temperature 39.3°C. Weight 33 pounds.

Nov. 15. 9 a.m. Dog is severely intoxicated. Ether anesthesia. Killed. The readings for non-coagulable nitrogen and urea nitrogen are given in Table II.

*Autopsy.*—Thorax, lungs, and heart normal. The viscera show nothing of interest except a slight congestion. The peritoneum is dry and clean. There are a few fibrinous adhesions about the site of obstruction. Intestinal tract below obstruction is collapsed and mucosa normal. The intestine above the obstruction shows only slight dilatation. There is no congestion of the mucosa and no ulceration. The stomach shows some congestion of the cardia. The material above the obstruction consists of thin yellow material, like pea-soup. 160 cc. in amount. Kept for further study.

The protocol of Dog 16-45 gives a typical story of an uncomplicated, untreated case of simple obstruction of the middle of the small intestine. Vomiting is constant, but the grave intoxication does not appear until the 5th day, when the non-coagulable nitrogen is found to be high—90 mg. per 100 cc. of blood. The autopsy findings are characteristic, and the peritoneum is clean.

Table II shows a considerable variety of non-coagulable nitrogen readings. The last three experiments show a definite rise in non-coagulable nitrogen above normal, and this is the rule. The first experiment, however, is a good example of a dog which maintained practically a normal level in spite of a prolonged obstruction. It should be kept in mind that this dog had a resistance considerably above normal, consequently was not as acutely intoxicated, and was killed 8 days after operation while still in fair condition. A similar state of affairs is noted in closed loops of the intestine, and with a slowly progressing intoxication the non-coagulable nitrogen may scarcely rise above its normal level.

This table of simple obstruction experiments gives also the urea nitrogen of the blood. It is noted at once that these intoxications give different urea readings with high non-coaguable nitrogen than is found in chronic nephritis. Urea nitrogen in nephritis usually

constitutes more than 50 to 60 per cent of the total nitrogen, but here it is seen that it often falls below 50 per cent. The residual or undetermined nitrogen in these experiments is constantly high. We suspect that this is a fairly constant feature in various proteose intoxications.

TABLE III.

*Closed Loop of Duodenum and Jejunum. Gastro-Enterostomy.*

Dog. No.	Day after operation.	Non-coagulable nitrogen.*	Remarks.
15-2	2	41	Condition good, vomiting.
	3	89	" "
	4	113	Grave intoxication; subnormal temperature. Killed.
15-9	1	51	Blood taken at time of operation. Weight 33 lbs.
	2	103	Dog sick, listless, vomiting. Died during night.
15-10	1	20	Blood at operation. 23 lbs.
	2	35	Dog toxic. Pulse good.
	3	70	Dog in fair condition. Intoxication moderate.
	4	135	Dog toxic. Pulse weak. Killed.
15-14	1	40	Blood before operation. 30 lbs.
	2	33	Dog vomiting. Slightly toxic.
	3	37	" " Moderately toxic. 28.5 lbs.
	4	53	Little vomiting. Condition the same.
	5	55	11 a.m. Dog sick. Muscular tremors. 26.5 lbs.
	5	65	2.30 p.m. Killed. Amino nitrogen 5.7 mg. per 100 cc.

\* Non-coagulable nitrogen is given in terms of mg. per 100 cc. of blood.

*Long Loop of Duodenum and Jejunum. Gastro-Enterostomy.*

*Dog 15-10.*—Long haired mongrel, male; weight 23.5 pounds.

Mar. 3. Isolated a long loop including lower half of duodenum and upper part of the jejunum; occlusion was effected by means of tapes applied as usual; gastro-enterostomy performed.

Mar. 4. Dog intoxicated. Temperature 38.5°C.

Mar. 5. Condition unchanged.

Mar. 6. Dog very sick; much vomiting. Pulse pressure low. Slow respiration. Temperature 37.3°C. Ether anesthesia and bleeding from carotid, which showed an arterial pressure scarcely above the usual venous pressure.

*Autopsy.*—Thorax normal. Peritoneum is clean and dry, except for a few bits of fibrin close to the site of operation. Liver shows a little congestion.

Other organs are normal. The loop is completely isolated from the intestine; it contains about 50 cc. of creamy, white, syrupy material having the characteristic odor. The mucosa shows a very slight amount of congestion, no ulceration, and is intact throughout. Under the microscope it appears normal. Loop fluid preserved for further study.

The protocol of Dog 15-10 (Table III) is a good example of uncomplicated closed intestinal loops of a certain type. It will be seen that the non-coagulable nitrogen of the blood rises regularly with the intoxication developing under these conditions. It must not be forgotten that these loops include the lower half of the duodenum and much of the upper jejunum. The ends of the loop are closed by tapes, and the contents of the upper duodenum must be forced back into the stomach where they gain access to the jejunum by a posterior gastrojejunostomy. This experiment, therefore, causes an obstruction to the first half of the duodenum, and this is of no small importance, as is seen on comparing these experiments with other closed loops of a different sort in Table V. We intend to take up this point in another communication.

TABLE IV.

*Closed Loop of Jejunum. Gastro-Enterostomy.*

Dog No.	Day after operation.	Non-coagulable nitrogen.*	Remarks.
15-29		mg.	
	2	23	Blood at end of infusion. Intoxication definite. Infusion of 1,000 cc. of 1% dextrose solution. Weight 35 lbs.
	4	35	Vomiting. Pulse fair.
	5	39	Dog weak. Blood taken before infusion. 1,000 cc. of 10% dextrose. 32 lbs.
	6	98	5 p.m. Infusion of 1,000 cc. of 10% dextrose. 11 p.m. Animal moribund. Killed. Loop rupture.
15-45	4	30	Dog slightly intoxicated. 22 lbs.
	5	76	10 a.m. Dog about the same.
	5	115	5 p.m. Dog looks sick. Pulse weak. Killed. Early peritonitis.
15-19	1	31	Blood at end of operation. 22 lbs.
	2	62	Dog very quiet. Died during night. Acute intoxication.

\* Non-coagulable nitrogen is given in terms of mg. per 100 cc. of blood.



*Long Loop of Jejunum with Rupture and Peritonitis.*

*Dog 15-29.*—Adult spaniel, male; weight 35 pounds.

Apr. 23. Isolated a long loop including the lower portion of the duodenum and about 3 feet of the jejunum, using tapes. Gastrojejunostomy performed as usual.

Apr. 24. Dog is sick. There is much dark brown vomitus. Pulse poor, and for this reason infusion of normal salt solution plus 1 per cent dextrose was given intravenously, followed by much improvement.

Apr. 25. Intoxication not striking.

Apr. 26. Much vomiting. Weight 33 pounds. Temperature 38.5°C.

Apr. 27. Dog seems sick. Weight 32 pounds. Temperature 38.4°C. 12 m. Infusion of 1,000 cc. of 10 per cent dextrose. 5 p.m. Dog not much improved. Given a second infusion of 1,000 cc. of 10 per cent dextrose.

Apr. 28. Much vomiting. Pulse tension low. Temperature 38.4°C. Weight 31.3 pounds. 5 p.m. Infusion of 1,000 cc. of 10 per cent dextrose. Dog is vomiting constantly. 11 p.m. Dog very sick. Ether anesthesia. Killed.

*Autopsy.*—The peritoneal cavity contains 500 cc. of blood-tinged loop fluid of the usual appearance. The peritoneum is specked with ecchymoses, but there is no fibrin. Rupture took place at the site of the lower ligature, which cut through the wall of the intestine. Upper ligature is tight. Death took place promptly from absorption of the toxic material poured from the loop into the peritoneum. The organs show some congestion associated with intoxication by means of loop fluid. Loop is empty. It shows congestion and some areas of submucous hemorrhage due undoubtedly to acute distention.

The protocol of Dog 15-29 (Table IV) shows the same rise in non-coagulable blood nitrogen noted in the closed intestinal loop experiments of Table III. These experiments show various complications met with in these loop experiments,—peritonitis, rupture of loop, and overwhelming intoxication. When the intoxication is very acute and severe, the infusion of normal salt or dextrose solutions will not depress the level of non-coagulable nitrogen in the blood.

*Long Loop of Jejunum. Rupture in 20 Days.*

*Dog 16-22.*—Fox-terrier, male; weight 18.5 pounds.

Sept. 28. Isolated a long loop of jejunum; ends of loop sectioned and turned in; the jejunum joined around the loop by end to end anastomosis to establish direct continuity of intestinal lumen.

Sept. 29. Dog looks well; no vomiting. (See Table V for details of temperature, weight, and non-coagulable and urea nitrogen). There was steady loss of weight during the next week with occasional attacks of vomiting; at times the dog eats a little food.

**TABLE V.**  
*Long Loop, Jejunum, Ileum.*

Dog No.	Day after operation.	Non-coagulable nitrogen.*	Urea nitrogen.*	Weight.	Temp.	Remarks.
		mg.	mg.	lbs.	°C.	
16-20	1	49	9	39.0	—	Before operation. Long loop jejunum.
	1	50	8	—	—	After operation.
	3	60	4	—	40.0	Condition good.
	4	28	7	—	40.0	Dog has vomited.
	5	35	11	37.5	40.1	No vomiting.
	8	24	8	36.8	40.1	" "
	9	22	6	37.3	—	Condition improved.
	10	28	8	37.5	39.8	Dog in good condition.
	11	28	8	36.5	39.7	" " " "
	13	33	12	35.3	—	Dog vomiting and intoxicated.
	14	53	16	—	—	Dog vomiting. Infusion of 1,000 cc. of 7% dextrose solution.
	15	51	18	33.0	39.3	Dog vomiting, weaker. Death 18th day.
16-22	1	22	10	18.5	—	Before operation. Long loop jejunum.
	3	36	8	18.0	39.4	Intoxicated. No vomiting.
	4	—	16	17.5	—	Vomiting, large amount.
	5	35	19	17.3	39.3	Little vomiting.
	7	30	14	15.8	38.9	" " Loss of strength.
	9	36	10	15.5	39.1	No vomiting.
	11	35	12	15.5	39.1	Solid feces.
	14	36	6	15.3	—	Condition the same.
	15	48	9	15.0	—	No vomiting.
	16	43	10	14.5	—	Animal looks intoxicated.
	20	41	20	13.7	38.8	Dog weak. Infusion of 1,000 cc. of 7% dextrose.
	21	64	29	—	—	Dog sick. Killed.
16-37	5	90	21	27.3	38.9	Vomiting for past 3 days. Long loop of ileum.
	6	45	20	27.3	39.1	Dog improved; no vomiting; eating.
	10	53	20	25.8	38.9	Some diarrhea.
	11	—	13	—	—	Dog looks well.
	13	59	21	24.3	—	Condition the same.
	14	57	25	24.0	—	Dog improved.
	15	31	19	23.8	—	Passed solid stool. Eating.
	17	54	32	24.0	38.9	Peristalsis visible in abdomen.
	18	40	19	24.0	39.3	Dog losing ground.
	19	40	9	23.8	38.9	Condition good. Killed.

\* Non-coagulable and urea nitrogen are given in terms of mg. per 100 cc. of blood.

Oct. 14. Dog has passed formed stools. No vomitus. Weight 14.5 pounds. There is evidence of chronic intoxication. Dog was injected with a toxic proteose obtained from ox pancreas; dog was slightly intoxicated by this proteose, while a normal control animal was fatally poisoned in 11 hours by a similar dose.

Oct. 15. Dog looks well; no vomitus.

Oct. 18. Dog is very sick. Ether anesthesia. Killed.

*Autopsy.*—Thorax, heart, and lungs normal; spleen, kidney, pancreas, etc., normal; liver rather atrophic; no signs of fatty degeneration. Peritoneal cavity contains a good deal of the loop fluid which has escaped from a recent rupture. There is little reaction in the peritoneum as the intoxication was so acute. Stomach and intestine outside of loop show congestion of the mucosa so common in proteose intoxication. Loop is made up of two parts isolated by old adhesions. One-half is slightly collapsed due to escape of fluid into the peritoneum; there is an ulcer in the wall, which has perforated the mucosa; all this portion is somewhat red and swollen, and shows two other ulcers involving the mucosa. The other half of the loop consists of three small coils twisted about its mesenteric attachment; adhesions have caused some constriction of mesenteric vessels giving engorgement of the loop but not infarction; this portion of the loop contains a pale, slate colored fluid with no blood; the mucosa is swollen and engorged but shows no hemorrhage; the walls of the loop are everywhere hypertrophied.

The protocol of Dog 16-22 (Table V) shows a different type of closed intestinal loop with a slowly progressing intoxication and relatively slight changes in the non-coagulable nitrogen of the blood. The loops of the jejunum or ileum are isolated completely by cross section of the gut in two places. The loop is made by turning in the ends of the isolated portion of the intestine or by doing an end to end anastomosis thus forming a circle out of the isolated gut. The continuity of the rest of the intestine is established by means of an end to end anastomosis which gives an unobstructed flow from duodenum to jejunum and ileum. This does away with the obstruction in the first half of the duodenum which is present in the loops isolated by ligature and gastrojejunostomy (Tables III and IV). The difference in severity of intoxication under these conditions is obvious.

The urea nitrogen readings in general show a low percentage value of the total non-coagulable nitrogen. The weight curve shows the gradual loss in body weight due to the chronic intoxication, even in the absence of vomiting.

The immunity to proteose injection (Dog 16-22) shown by a dog with a long standing closed intestinal loop indicates that the presence

of a closed loop of intestine causes a chronic proteose intoxication which gives a certain degree of immunity against poisoning by various foreign proteoses. This point will be taken up in another communication and the experiments will be given in detail.

TABLE VI.  
*Long Loop, Jejunum, Ileum, Complications.*

Dog No.	Day after operation.	Non-coagulable nitrogen.*	Urea nitrogen.*	Weight.	Temp.	Remarks.
		mg.	mg.	lbs.	°C.	
16-42	3	32	13	21.0	39.1	Long loop jejunum, obstruction, peritonitis.
	4	39	15	20.5	39.0	Considerable vomiting.
	5	39	13	20.7	38.8	" " Animal looks sick.
	6	50	22	21.0	37.9	9.30 a.m. No vomitus.
	6	56	39	—	34.5	4 p.m. Dog toxic. Killed.
16-48	4	30	14	19.5	39.0	Long loop jejunum, terminal obstruction, and volvulus.
	6	45	19	19.0	39.2	Condition good. No vomiting.
	8	32	15	19.3	38.8	Condition the same.
	12	46	22	19.5	39.3	" " " Found dead on 13th day.
16-57	1	58	23	18.5	—	Short loop of ileum. General peritonitis.
	6	44	11	15.5	39.3	Dog has been in good condition.
	8	166	51	15.5	36.8	Some vomiting. Animal moribund. Killed.
16-71	1	36	12	27.5	—	Long loop of jejunum. General peritonitis.
	2	36	12	26.3	38.3	Dog in fair condition.
	4	53	31	—	—	Animal moribund. Killed.
16-39	1	—	15	24.5	—	Long loop of jejunum. General peritonitis.
	3	71	18	23.5	38.8	Much vomiting. Pulse poor. Infusion of 1,000 cc. of 5% dextrose. Blood at end of infusion. Death next day.

\* Non-coagulable and urea nitrogen are given in terms of mg. per 100 cc. of blood.

*Long Loop of Jejunum. Obstruction. General Peritonitis.*

*Dog 16-42.*—Mongrel, male; weight 22 pounds.

Nov. 6. Isolated a long loop of jejunum by section and inversion of ends; jejunum joined around the loop by means of end to end anastomosis between upper and lower end.

Nov. 8. Dog pretty well. Temperature 39.2°C. Weight 22 pounds.

Nov. 9. Much vomiting.

Nov. 10. Dog seems better. (See Table VI for details.)

Nov. 11. Little vomiting. Temperature 37.9°C. Weight 21 pounds. 4 p.m. Dog is very weak. Temperature 34.5°C. Ether anesthesia. Killed.

*Autopsy.*—Thorax, heart, and lungs normal. Peritoneal cavity contains many isolated pockets of purulent exudate. A large round worm is free in the peritoneal cavity, evidently having escaped from the loop, which contains numerous similar worms. There are organized adhesions just below the end to end anastomosis causing a sharp kink and probably complete obstruction. Duodenum and jejunum contain the usual obstruction fluid. The loop contains 130 cc. of pale, slate colored fluid with a strong odor. There are numerous live and active round worms in the loop. No point can be found where the worm escaped from the loop. The peritonitis is probably of 1 or 2 days' duration.

The protocol of Dog 16-42 (Table VI) shows some of the complications which may arise in the closed loop experiments. Peritonitis is most common, but volvulus and obstruction may occur. These complications do not modify the picture, and we have good evidence that peritonitis alone may be associated with a high non-coagulable nitrogen. It is of interest to note that we have been able to isolate a toxic proteose from peritoneal exudates, and we believe that this proteose is of considerable importance in explaining the intoxication of general peritonitis. The rise in non-coagulable nitrogen in the blood may be in part due to the proteose intoxication.

*Proteose Injection. Rise in Non-Coagulable Nitrogen.*

*Dog 15-50.*—Small black and tan, male; weight 13 pounds.

12 m. (A) Blood non-coagulable nitrogen 33 mg. per 100 cc. of blood; ether anesthesia and intravenous injection of purified proteose obtained from closed intestinal loops. Kymograph observation during slow injection of 65 cc. of solution of proteose which had been reprecipitated first by alcohol and then by half saturation with ammonium sulphate as described previously (5). There was a little fall in blood pressure.

12.45 p.m. After injection (B) blood non-coagulable nitrogen 40 mg. per 100 cc. of blood.

3.15 p.m. (C) Blood non-coagulable nitrogen 80 mg. per 100 cc. of blood.

4 p.m. Death with prostration and subnormal temperature.

*Autopsy.*—There is the typical splanchnic engorgement due to fatal proteose intoxication. Spleen and liver are swollen and purple. Mucosa of duodenum and jejunum is swollen and deep reddish purple.

*Dog 15-51.*—Small fox-terrier, female; weight 15.5 pounds.

12 m. (A) Blood non-coagulable nitrogen 39 mg. per 100 cc. of blood. Ether anesthesia and intravenous injection of purified proteose obtained from closed intestinal loops. Kymograph observation during injection of 50 cc. of fluid showed no change in blood pressure.

12.30 p.m. After injection (B) blood non-coagulable nitrogen 32 mg. per 100 cc. of blood.

5 p.m. (C) Blood non-coagulable nitrogen 58 mg. per 100 cc. of blood.

Dog is prostrated; much vomiting and diarrhea.

Died in night.

*Autopsy.*—The findings are characteristic of proteose intoxication. There is much blood-tinged fluid in the intestines, and the mucosa is red and swollen.

These two experiments (Dogs 15-50 and 15-51) are of considerable interest, and show that acute poisoning with a pure proteose may cause the blood non-coagulable nitrogen to double in amount in 3 hours; for example, a rise from 40 to 80 mg. The proteose used in these experiments was pure, and not over 200 mg. were injected. The addition of this to the blood itself could not be detected by any method in use, and the method used by us causes precipitation of all primary proteoses at least. The great rise in non-coagulable nitrogen obviously must be explained by disintegration of body or tissue protein. This is of importance in explaining the high non-coagulable nitrogen associated with the closed loops of intestine or intestinal obstruction. In both instances we are dealing with a proteose intoxication, and we believe that much of the increase in blood non-coagulable nitrogen is due to disintegration of the tissues of the body. Catabolism, in other words, must be responsible for much of the non-coagulable nitrogen rather than retention.

Tables VII and VIII give the nitrogen partition of the total non-protein nitrogen of the blood expressed in mg. per 100 cc. of blood. One control nephritis gives a residual nitrogen of 11 per cent and 82 per cent urea nitrogen. A second case (Dog 16-49 in Table VIII) shows a similar picture. This experiment presents isolation of the bladder and implantation of the ureters into the intestine. There

TABLE VII.

*Intestinal Loops. Peritonitis and Obstruction.*

No.	Non-coagulable nitrogen.			Urea nitrogen.		Amino nitrogen.		Uric acid nitrogen.		Creatine nitrogen.		Creatinine nitrogen.	Residual nitrogen.		Remarks.
	mg.	mg.	per cent	mg.	mg.	mg.	mg.	mg.	per cent						
Dog 16-37	40	9	23	1.4	0.6	0.6	1.5	26.9	67	Long loop of ileum. Killed 19th day.					
“ 16-42	56	39	69	4.5	0.6	1.0	5.6	5.3	9	Long loop of jejunum. Peritonitis, obstruction.					
“ 16-57	166	51	30	6.0	0.6	0.8	11.0	96.6	58	Long loop of ileum. Peritonitis.					
“ 16-71	53	31	58	3.7	1.1	0.8	7.3	10.1	19	Long loop of jejunum. Peritonitis.					
“ 16-80	40	28	70	3.9	1.0	0.3	3.1	3.7	9	Long loop of ileum. Peritonitis.					
“ 15-45	115	77	60	—	—	0.8	5.9	—	—	Long loop of jejunum.					
Cat 16-7	296	137	46	6.8	2.3	0.7	21.3	127.9	43	Long loop of jejunum and obstruction. Death in 4 days.					
Dog 16-45	90	43	47	5.5	—	—	—	—	—	Simple obstruction. Death in 5 days.					
“ 16-82	47	17	36	2.5	1.4	0.2	3.3	22.6	48	Simple obstruction. Killed in 7 days.					
Case 1 (Man)	164	107	65	2.3	0.9	0.3	10.2	35.2	21	Simple obstruction. Pneumonia. Death in 5 days.					

Nitrogen is given in terms of mg. per 100 cc. of blood.

was some obstruction to the outflow of urine and some escape into the peritoneum with an irritant peritonitis and absorption consequent to this. Here there are two factors—retention or lack of elimination plus peritonitis.

The tables show a high per cent of residual nitrogen or a low relative per cent of urea nitrogen. This is true particularly in the severe proteose intoxication with high non-coagulable nitrogen. Similar high readings in cases of nephritis and retention will scarcely show such high percentages of residual nitrogen. For this reason we be-

TABLE VIII.

*Bladder Isolation, Chronic Nephritis, Peritonitis, etc.*

							gm.	Creatinine nitrogen.			
							g.	mg.	mg.	per cent	
							4	7.1	28.9	22	Bladder isolation. Urine es- cape. Peritonitis.
							0	12.0	41.9	11	Chronic nephritis. Old dog. Hemorrhagic gastritis.
							5	1.1	23.2	11	Acute distemper.
							6	5.2	9.1	25	Drained loop of ileum. Sub- cutaneous abscess.
							6	3.8	16.3	40	Skin incisions. Mild dis- temper.
16-69	164	58	38	4.2	1.2	0.9	5.3	94.4	57	General peritonitis.	
16-85	67	29	43	3.1	0.4	0.7	3.6	30.2	45	Distemper. Pneumonia.	

Nitrogen is given in terms of mg. per 100 cc. of blood.

lieve that the non-coagulable nitrogen determination gives more information than does urea nitrogen, and is of more clinical value in diagnosis and prognosis.

The blood content of amino nitrogen and uric acid nitrogen is low normal, and shows fluctuations within normal limits. Ammonia nitrogen was done in the majority of experiments but the method is inaccurate and the values are probably too high. It seemed best to leave these analyses out of the table and assume that ammonia nitrogen is included in the urea nitrogen. The difference scarcely exceeds 1 mg. at the most.

The creatine fraction is constantly low, but it is of interest to note that the creatinine nitrogen may be very high in many of these proteose intoxications, particularly in intestinal obstruction and closed intestinal loops. There are many readings of more than 5 mg. per 100 cc. of blood, and one experiment (Table VII, Cat 16-7) shows a reading of 21.3 mg. This occurred in a cat with intestinal obstruction plus a closed intestinal loop with a non-coagulable nitrogen of 296 mg. per 100 cc. of blood. The kidneys in all these cases,



unless otherwise noted, were normal. It is to be recalled that all these animals were sick and refused food—in fact the majority were vomiting more or less. A human case of intestinal obstruction and pneumonia shows a very high creatinine value, 10.2 mg., but autopsy showed practically normal kidneys.

### *Human Intestinal Obstruction. Death in 5 Days.*

*Case I.*—C. K., German, male, 68 years of age.

*Past History.*—Negative.

*Present Illness.*—Began Jan. 16, 1916, with indefinite pain. No bowel movements since this time.

Jan. 17. A good deal of epigastric pain with vomiting, which became fecal in type the following day. Patient given castor oil and salts without results.

Jan. 18. Condition unchanged, except that vomiting became more severe.

Jan. 19. Entered hospital. At this time he had marked abdominal tenderness, and was vomiting frequently. Vomitus fecal in odor, watery, with fine brownish precipitate. Blood obtained showed very high non-coagulable nitrogen. Patient given 900 cc. of 6 per cent glucose with sodium carbonate intravenously. Infusion improved condition of patient.

*Blood Examination.*—White blood cells 18,400; hemoglobin 90 per cent.

3 p.m. Laparotomy and abdominal exploration. A portion of greatly congested intestine, about 10 cm., was found; the wall was edematous. It was placed back in the abdominal cavity. Abdomen closed. Volvulus(?).

Jan. 20. 10 a.m. Blood again obtained, and showed some decrease in non-coagulable nitrogen; vomiting not so marked; considerable fecal matter obtained by means of an enema. Pulse not good. 4 p.m. Blood again obtained; showed rising non-coagulable nitrogen. Given infusion of 750 cc. of 6 per cent glucose intravenously.

Jan. 21. 6 a.m. Patient died in stupor. Blood obtained by cardiac puncture a few minutes after death.

Autopsy performed 5 hours after death.

*Anatomical Diagnosis.*—Intestinal obstruction; volvulus (?); operation wound for relief of obstruction; early infarction and necrosis of loop of ileum; early serofibrinous peritonitis; bronchopneumonia (pseudolobar) of both lungs; acute hemorrhagic bronchitis; cloudy swelling of viscera.

The abdomen is considerably distended and tense. On incision a small amount of blood-tinged, slightly turbid fluid is found between the coils of an enormously distended small intestine. The loops are somewhat glued together by dry, plastic exudate. The surface of the intestines is somewhat dry and very definitely injected, more especially in the region of the abdominal incision. There are no adhesions except over the spleen. There are no hernial openings to be found in the pelvis or inguinal region. One of the stitches in the abdominal wound has caught

and firmly held a bit of omentum. One segment of the intestine, dark red in color and considerably swollen, measuring about 15 cm. in length, is found close to the liver. The swelling involves the wall of the intestine and the mesentery to a distance of about 5 cm. from the intestinal attachment. There is a clean-cut line of demarcation between the relatively normal but elongated mesentery and the short, inflamed, edematous mesenteric portion close to the intestine. The line of demarcation on the intestine is quite sharp, particularly at the upper end. It appears as though a band, or definite tight constriction, had been drawn about the portion of the ileum including the small part of the mesentery, shutting off a considerable part of the blood supply. This may have been due to a twist of the relaxed elongated mesentery. This was evidently relieved at operation, but the circulation did not establish itself properly owing to tissue injury, and the general appearance was that suggesting early hemorrhagic infarction. The picture, however, was not complete. Careful section of the mesenteric veins showed them to be quite free from thrombi even in their finer branches. There may be some very small thrombi in the smaller branches close to the mesenteric border, but these could not be dissected out.

*Lungs.*—The right lung weighs 870, and the left 560 gm. The lungs are voluminous, cushiony in their anterior portions; they are heavy and consolidated posteriorly. The pulmonic vessels are clear. The bronchi are intensely inflamed, their mucosa is velvety and purple, and they contain much serous blood-tinged fluid. On section the anterior portions are dry and cushiony. The posterior portions are consolidated and very moist. One can scrape off purulent material. The consolidation involves the posterior portion of the left upper and the greater portion of the right upper lobe and part of the right middle lobe. These areas of consolidation are mottled gray and purplish red. Some of the gray areas are very soft, and creamy material can be scraped off, indicating a beginning resolution of the exudate. This pneumonic process must have been of several days duration—estimated 2 to 3 days.

*Kidneys.*—Capsule comes off easily leaving a smooth surface, but for two retention cysts. There is one large retention cyst in the upper pole of the right kidney measuring about 2 cm. in diameter. Its wall contains a few thin plaques of calcified material. The kidney parenchyma on section appears normal. The pelvis is normal.

*Microscopic Examination. Kidneys.*—There are a few hyaline casts in some of the tubules, also a few hyaline scars in the cortex. In general the parenchyma looks normal except for definite cloudy swelling of the epithelium lining the convoluted tubules. There is no epithelial necrosis. The stroma of the pyramids shows a little edema.

*Lungs.*—The patches of pneumonia show the alveoli filled with an exudate of coagulated serum, mono- and polynuclear cells, fibrin, and enormous numbers of bacteria. In places the bacteria form almost a solid mat of rods showing capsules. The great overgrowth of bacteria is the striking feature of this lung.

*Mesentery.*—The swollen hemorrhagic area shows a few small recent thrombi, but most of the veins are free. There is extreme extravasation of red blood cells into its stroma. The other portion of the anatomical protocol may be omitted, as it has no bearing on the points under consideration.

TABLE IX.  
*Human Intestinal Obstruction.*

Time.	Non-coagu- lable nitro- gen.*	Urea nitro- gen.*	Urea.	Remarks.
	mg.	mg.	per cent	
2 hrs. before operation.	145	30	20	After infusion of 900 cc. of 1% sodium carbonate and 6% glucose.
18 " after "	76	48	63	After infusion of 400 cc. of 6% glucose.
26 " " "	80	52	60	" " " 750 " " 6% "
46 " " "	164	107	65	Heart blood (see Table VII).

\* Non-coagulable and urea nitrogen are given in terms of mg. per 100 cc. of blood.

The human case (Table IX) is of much interest as the observations on the blood are fairly complete, and autopsy material is available. Clinically it was a clear case of obstruction due to volvulus with partial infarct formation in the intestine and a complicating pneumonia. The non-coagulable nitrogen of the blood was constantly high in spite of transfusion at various times. The elimination of urine was considerable, because of the transfusions, and showed nothing of interest—a mere trace of albumin and an occasional cast.

Table IX shows that there is a rise in urea and drop in non-coagulable nitrogen after the first infusion. We have noted this under the same circumstances in dogs, but are not able to advance an explanation. The high creatinine value (Table VIII) is of interest, as the kidneys are shown to be practically normal. This case gives blood findings identical with those observed in animals under similar conditions, and indicates that our experiments give information which may be of value in the study of human cases.

#### DISCUSSION.

We believe that it may be assumed as proven that the non-coagulable nitrogen of the blood in the majority of instances is definitely

increased in intestinal obstruction or with closed loops of intestine. With acute intoxication this rise in non-coagulable nitrogen is apt to be more striking and constant. When this rise in non-coagulable nitrogen of the blood does occur, it is a grave sign, and is a clinical index of a severe intoxication even in spite of other clinical evidence to the contrary. But a low non-coagulable nitrogen does not guarantee a mild grade of intoxication. We are convinced that a knowledge of the blood non-coagulable nitrogen is of considerable clinical value in the prognosis of acute abdominal conditions.

It should be kept in mind that the urea nitrogen as well as creatinine nitrogen may show high values in these conditions, and these points may be of value in differential diagnosis. It is established that other conditions besides chronic nephritis may show a marked increase in the creatinine and urea nitrogen of the blood. It should be recalled that "creatinine rises above 2.5 mg. per 100 cc. of blood almost without exception only in conditions with renal involvement" (Myers and Lough (6)). The conditions studied by us show a high creatinine fraction and constitute exceptions to this statement.

It will be noted that the undetermined nitrogen in these experiments is unusually high—more so than in cases of nephritis with high non-coagulable nitrogen. This may be a peculiarity of this type of intoxication as contrasted with simple retention of nitrogenous material, and a study of this point may bring out much valuable information.

Having established the fact that the non-coagulable nitrogen of the blood is much increased in many cases of intestinal obstruction or of closed intestinal loops, we may now ask: Why does not the kidney eliminate these substances immediately? The kidneys are normal in gross and by functional tests in practically all cases. There can be no true retention because of impaired kidney function. There may be two or more factors concerned. We know that in intestinal obstruction the current of fluid is mainly out of the body and by way of the intestinal tract, and it is possible that this favors the accumulation of certain products in the blood stream. The kidneys excrete small amounts of highly concentrated urine.

It is to be remembered, too, that injection of a small amount of a toxic proteose may cause a great rise in non-coagulable nitrogen in

the blood; for example, a rise from 40 to 80 mg. in 3 hours. This cannot be due to lack of elimination, and we must assume destruction of body protein to account for this remarkable change. We may assume that any acute proteose intoxication may be associated with a similar rapid rise in non-coagulable nitrogen in the blood. When we have more information about this point, we may better understand the manner in which the toxic proteoses injure the body and perhaps the various methods of body defense.

General peritonitis is often associated with a definite rise in non-coagulable nitrogen of the blood. How may we explain this observation? It may be argued that paralytic ileus is alone responsible, and this may be true in part. However, we think it important that a toxic proteose can be isolated from the exudate in cases of general peritonitis, and obviously must be absorbed by the host. The proteose intoxication may well be responsible for this change in non-coagulable nitrogen. We hope to report further on this point in the near future.

#### SUMMARY.

Intestinal obstruction, as a rule, is associated with an increasing amount of non-coagulable nitrogen in the blood. With acute intoxication the rise in non-coagulable nitrogen may be rapid and reach as high as three or even ten times normal. With more chronic intoxication there may be little or no rise in the blood non-coagulable nitrogen.

Closed intestinal loops show exactly the same picture, and, when combined with obstruction, may give very high nitrogen readings.

Acute proteose intoxication due to injection of a pure proteose will show a prompt rise in blood non-coagulable nitrogen, even an increase of 100 per cent within 3 or 4 hours.

These intoxications also show a high blood content of creatinine and urea. The residual or undetermined nitrogen may be very high.

A human case of intestinal obstruction with autopsy presents blood findings exactly similar to those observed in many animal experiments.

Clinically the non-coagulable nitrogen of the blood may give information of value in intestinal obstruction. A high reading means

a grave intoxication, but a low reading may be observed in some fatal cases and gives no assurance that a fatal intoxication may not supervene.

The kidneys in practically all these experiments are normal in all respects.

It is possible that protein or tissue destruction rather than impaired eliminative function is responsible for the rise in non-coagulable nitrogen of the blood in these acute intoxications.

Transfusions of dextrose solutions often benefit intestinal obstruction, and may depress the level of the non-coagulable nitrogen in the blood. Some cases show no change in non-coagulable nitrogen following transfusions and diuresis, and, as a rule, such cases present the most severe intoxication.

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# THE ORIGIN AND STRUCTURE OF A FIBROUS TISSUE FORMED IN WOUND HEALING.

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PLATES 106 TO 109.

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## INTRODUCTION.

In a previous paper<sup>1</sup> the author showed that in living cultures of adult frog tissues there occurs, in many instances, a transformation of the plasma clot in which the living tissue is imbedded. This transformation results in a consolidation or fusion of the elements of the fibrin net and a consequent formation from it of a fibrous tissue which is identical in its form and structure and in many of its staining reactions with a regular collagenous connective tissue. It was felt that, whether or not this new fibrous tissue directly formed from the fibrin clot remained as a permanent connective tissue, such a reaction must play a fundamental part in the processes attendant upon wound healing. The present paper<sup>2</sup> gives the results obtained from an extensive series of experiments undertaken for the purpose of studying the action and fate of the fibrin clot formed during wound healing, and they give evidence that in the healing of skin wounds in the frog a definite transformation of the fibrin clot takes place such as was found to occur in the tissue cultures. This transformation also results in the formation of a new fibrous tissue, without intracellular action, which is apparently identical with regular permanent connective tissue.

<sup>1</sup> Baitsell, G. A., The Origin and Structure of a Fibrous Tissue Which Appears in Living Cultures of Adult Frog Tissues, *Jour. Exper. Med.*, 1915, xxi, 455.

<sup>2</sup> A report of this work was given at the meeting of the American Association of Anatomists held at New Haven, Conn., Dec. 28-30, 1915. An abstract appears in the proceedings in the *Anat. Rec.*, 1916, x, 175.

*Material and Methods.*

It was thought best to carry on the experiments in wound healing with the frog inasmuch as the tissues used in the previous work with living tissue cultures had been obtained from this animal. During the present experiments thirty-one animals were used and a total of fifty operations were performed. The process of wound healing has been studied at various stages both in the living animals and in the preserved material. For the study of the preserved material pieces of the tissues containing the wounds were removed at various times after the operations. These were preserved in Zenker's solution, imbedded in paraffin, and sections, in various planes, were made at 10  $\mu$ . In general the stain used has been Mallory's connective tissue stain modified according to Mall.<sup>3</sup> Mallory's stain, unmodified, and the Van Gieson picro-fuchsin stain have also been used for comparison. A discussion of the staining reactions will be found in a later section of this paper.

In the preliminary experiments, the semimembranosus muscle lying on the dorsal surface of the hind legs of the frog was used. After anesthetization the animal was placed on an operating board dorsal surface up. The skin was then sterilized by washing it with a 0.001 per cent solution of mercuric chloride. This method of sterilization appears to be adequate for this work and there has been very little trouble from infection. An incision of about 5 to 7 mm. in length was then made in the median dorsal surface of either or both of the hind legs. The cut edges of the skin were separated and held apart and a cube of the underlying muscle tissue measuring about 2 mm. was removed. The cavity thus formed in the muscle tissue was either filled with plasma which had been obtained in the usual manner<sup>4</sup> from another frog, or by the blood and lymph which flowed into the wound from the cut edges. After the operation the frog was held in position for a few minutes until the clot had formed in the wound.

The results obtained from these experiments were not satisfactory. The many movements of the legs and the consequent expansion and

<sup>3</sup> Mall, F. P., On the Development of the Connective Tissues from the Connective-Tissue Syncytium, *Am. Jour. Anat.*, 1901-02, i, 338.

<sup>4</sup> Baitsell, G. A., The Origin and Structure of a Fibrous Tissue Which Appears in Living Cultures of Adult Frog Tissues, *Jour. Exper. Med.*, 1915, xxi, 456.



contraction of the muscle fibers resulted, in many cases, in a breaking up of the plasma clot in the wound cavity and its destruction, so that it was impossible to make a study of it. The friction of the overlying skin of the leg during these movements also tended to make the operations unsuitable for the present study of the fibrin clot. In a number of these experiments which were first made, stitches were taken in the skin to hold the cut edges together and in their proper positions. In the later operations it was found that this was not necessary for it was observed that when a cut was made in the skin, as noted above, if the animal remained quiet for a few minutes after the operation, the coagulation of the blood and lymph formed a tissue of sufficient strength to hold the cut edges of the skin in their proper position. It appeared evident from this that wounds in the frog skin would afford a suitable field for the study of the process of wound healing, particularly with regard to the action and fate of the fibrin clot. Later experiments showed that the skin lying on the posterior dorsal surface of the animal offered an even better field for these experiments than did the skin of the legs inasmuch as it is not subject to an almost continual, and at times violent strain as the result of leg movements. Wounds in both these regions have been studied and form the basis of the results given in the present paper.

*Observations on the Process of Wound Healing in the Skin  
of Living Frogs.*

The healing of various types of wounds has been observed in the living animals. In the first type of experiments, a simple incision was made in the skin of the frog either on the dorsal surface of the leg or on the back. As far as could be told by the observations on the living animal these wounds healed very quickly. The formation of the coagulation tissue as a result of the clotting of the blood plasma or lymph present in the wound occurred within a few minutes after the wound was made. As a result the cut edges were firmly held in position and the frog could then be placed in the aquarium without injury to the new tissue. The animals examined 24 hours later showed only slight evidence of the wound. The cut edges were still firmly held and all that could be noted was the light colored line of coagu-

lation tissue marking the extent of the incision. In the course of the next day or so the epithelial cells moved over this area and obliterated all evidences of the wound.

In the second type of experiments a piece of skin measuring about 5 by 2 mm. was detached on three sides. The fourth side (2 mm.) was left attached to the body. In this way a flap of skin was formed which was severed from the body except at one end. In such cases the coagulation tissue which quickly formed held all the cut edges of the skin and the loose flap in place, and thus an operation which one would naturally expect, without the aid of sutures to hold the edges in place, to have left a large open wound in the skin with a consequent slow healing was permanently closed within a few minutes after the operation. In the following days one could observe, as in the previous type of experiments, a gradual obliteration of the cuts by the epithelial cells which moved out over the coagulation tissue from the cut edges of the skin.

In the third type of experiments a piece of skin from the median posterior region of the back, measuring about 5 mm. square, was completely severed from the body of the animal, placed in sterile Ringer solution for a few minutes, and then replaced in the cavity from which it had previously been taken. The observations showed that no other support than the coagulation tissue which formed was necessary in order to hold such a piece of skin in position in the wound cavity, and the complete obliteration of the wound occurred in a few days by the movement of the epithelial cells over the coagulation tissue. The results of other experiments of this type showed that the living skin from another animal or a dead tissue, such as the stratum compactum obtained by pancreatin digestion of adult frog skin, could be transplanted into a wound cavity in the skin and retained there by the action of the coagulation tissue alone. In a later paper it is hoped that more complete results regarding such skin transplantation can be presented. The only fact regarding them which should be emphasized in the present paper is the action of the coagulation tissue in the wound cavities as shown by its ability to hold the transplant in position without the aid of sutures.

In the last type of experiments, a piece of skin, varying in size from 3 to 5 mm. square, was completely removed from the body. The resulting cavity was quickly filled with the coagulation tissue. In this

type of experiment it was found that in some cases, particularly if the cavity caused by the removal of a piece of skin was large, it was better to supply additional plasma obtained from another animal in order that the wound cavity might be completely filled. A wound of this type (Fig. 1) gives the best opportunity for the study of the coagulation tissue. It forms a hyaline mass in the wound cavity and is glossy and transparent, and until it has been covered by the epithelial cells which move in from the surrounding areas the underlying muscle tissue can be seen through it. This fibrin clot or coagulation tissue when it is first formed may sometimes be destroyed by extremely violent movements of the animal, especially if the wound be a large one. An increase in the strength of the new tissue soon becomes evident and in a few days it appears to be as immune from injury as regular skin tissue. As far as can be told by the observations on the living animals, this coagulation tissue formed by the clotting of the plasma in the wound remains permanently and takes the place of the piece of skin tissue which was removed. From this it is clear that in a skin wound in the frog, if the conditions are right, a new tissue can be formed almost immediately after the injury which is sufficiently strong to retain its place in the wound cavity and to hold the cut edges in position. The wound, to all appearances, is thus healed without waiting for a process of regeneration of tissues by cell division. Probably the most impressive part of this process of wound healing, to an observer, is the rapidity with which it takes place. The coagulation of the plasma closes the wound almost immediately and then, evidently by a transformation of this fibrin clot, a firm, resistant, and apparently permanent tissue is formed which serves as a regular connective tissue.

During the course of the experiments, observations have also been made on the healing of wounds in which no coagulation tissue was present. Such a condition may arise either because of the lack of a sufficient amount of plasma in the wound at the time it was made, because of a later destruction of the coagulation tissue as a result of tension caused by too vigorous movements, or because of an infection. In cases of this kind the process of wound healing in the skin of a frog is a comparatively long and slow one, and in some cases results in the death of the animal by a later infection received through the open wound.

*Study of the Prepared Material.*

In Fig. 1 a view of a wound in the skin of a frog is shown *in toto* at a magnification of about 15 diameters. This wound was removed from the animal and preserved when it was three days old. The wound, caused by the removal of a piece of skin, originally measured about 5 by 2 mm. The figure was drawn with the aid of a Zeiss binocular microscope from the under surface of the skin and shows the wound surrounded by the skin tissue and filled with the coagulation tissue (fibrin clot) which was formed within a few minutes after the wound was made by a clotting of the blood plasma and lymph. Several large blood vessels are shown, some of which run into the coagulation tissue. Some of these vessels appear to be enlargements of the vessels supplying the skin in that region, while others apparently arise in the underlying muscles. In some cases the wound is found to be attached to the muscles by a number of small vessels.

A transverse section of the same preparation is shown in Fig. 2 at a magnification of 77 diameters. At either end of this figure may be noted the cut ends of the skin which mark the boundaries of the wound. Connecting these can be seen the bridge of new tissue which has formed in the wound cavity. Lying above this coagulation tissue are several layers of epithelial cells which have wandered in from the skin tissue surrounding the wound. This movement of the epithelial cells begins very soon after the wound has been made and continues until, as shown in later stages (Fig. 6), they form a thick layer over the coagulation tissue. The epithelial cells in Fig. 2 are more numerous near the cut edges of the skin. At these points the coagulation tissue is drawn out into a rather thin strand which is attached to the cut edges of the skin and which serves to hold them in place. It can be noted in this figure that fibers are present in the coagulation tissue.

The observations on the living animals have shown that the coagulation tissue in a 3 day wound is very firm and resistant as compared with a young fibrin clot. From this it is evident that already some change has taken place in the clot and this fact can also be noted from the study of the prepared material. In Fig. 3 a portion of the coagulation tissue, from the same preparation as Fig. 2, is shown at

a magnification of 787 diameters. At this magnification one is able to see clearly the histological structure of the coagulation tissue. Quite large areas can be noted in this figure in which the fibrin net has not been changed but still retains its characteristic structure. In other regions, however, the fibrin net has been changed into a new fibrous tissue. In such areas it appears that there has been a fusion of the elements of the fibrin net to form long, wavy, fibrous bundles which are typical in structure and appearance to those found in various forms of connective tissues. This new fibrous tissue is apparently identical with that which forms in plasma clots in tissue cultures. The transformation of the fibrin clot in this wound into a new fibrous tissue has not been due to an intracellular action for, with the exception of some blood corpuscles which were present in the plasma when it clotted, some of which can be seen in Fig. 3, no cells of any kind are present in the new fibrous tissue except in the extreme edges where the new tissue is in contact with the cut edges of the skin. A thorough study of this point has been made in this and other similar preparations and it can be definitely stated that in wounds in the frog skin, as has previously been shown to be the case in tissue cultures, a direct transformation of a fibrin clot into a new fibrous tissue will occur without intracellular action.

In Fig. 4, which is a portion of a longitudinal section of a 4 day wound at a magnification of 787 diameters, a more complete transformation of the plasma clot is seen. The section from which this figure was drawn was taken from near the upper surface of the coagulation tissue; that is, from the dorsal portion of the clot lying just below the epithelial cells. At either edge of the coagulation tissue may be noted some of the epithelial cells which mark the boundary of the wound and which have grown down on both sides of the coagulation tissue. This downward growth of the epithelial cells can be noted in some of the transverse sections of the wound tissue (Fig. 2). A study of Fig. 4 shows that the fibrin net structure has disappeared and the new fibrous tissue has been formed which, as in Fig. 3, is composed of bands of fibrils showing the typical structure of regular connective tissue fibers. The new fibrous tissue is denser near the bottom of the figure and it is in this region that its structure is seen to the best advantage. All the coagulation tissue shown in

the figure has lost the original fibrin net structure, but in the less dense regions of this tissue, in which numerous vacuoles are present, the transformation into the new fibrous tissue has not been so complete. No fibroblast cells are present in this region of the clot and so in this case also the transformation of the fibrin clot into the new fibrous tissue has not been due to any intracellular action. In this preparation centrifuged blood plasma which had been obtained from another animal was placed in the wound to form the clot and this fact accounts for the almost complete absence of blood corpuscles. Those that are shown in this figure are in most cases matted together and are evidently undergoing degeneration.

In Fig. 5 is shown, at the same magnification as Fig. 4, another longitudinal section of the same preparation taken at a deeper level. In this figure a considerable number of both fibroblast cells and blood corpuscles are shown. In this figure a transformation of the fibrin clot has also occurred and a new fibrous tissue has been formed containing bundles of wavy fibers. These are particularly well shown to the left of the figure. In this region numerous cells are also present and as a result the bundles of fibers are more or less separated from each other. To the right of the figure fewer cells are present and the newly formed fibrous tissue is more closely massed together. The bundles of fibers, however, can be clearly seen.

A comparison of the new fibrous tissue shown in Figs. 3, 4, and 5, which has been formed through a transformation of the fibrin clot, with the fibrous tissue found in the plasma clot in living cultures of frog tissues, as noted in a previous paper,<sup>5</sup> shows that they are identical.

The conditions present in an older wound are shown in Fig. 6, which is a transverse section of a 12 to 13 day preparation at a magnification of 240 diameters. At either end of the figure can be seen the cut ends of the skin tissue which mark the boundaries of the wound and between these the new tissue which has been formed in the wound. Overlying this region are the epithelial cells which are piled up many layers thick. The wound cavity, except for a small portion

<sup>5</sup> Baitsell, G. A., The Origin and Structure of a Fibrous Tissue Which Appears in Living Cultures of Adult Frog Tissues, *Jour. Exper. Med.*, 1915, xxi, 478-79. Compare Fig. 3 of this paper with Figs. 11 and 17 of previous paper.

lying near the right end of the figure, is filled with the new fibrous tissue. Fibroblast cells are present all through the new tissue. In the region lying to the right a portion of the original fibrin clot apparently still remains. In Fig. 7, which was drawn from the same section as Fig. 6, this region of the wound is shown at a magnification of 787 diameters. The figure shows that the fibrin clot has lost its original structure, as shown for example in Fig. 3, and has become a more or less compact mass in which, at various places, it can be noted that fibers have formed. Around the edges of the fibrin mass, in particular, a great many of the bundles of fibers can be seen. These are attached to the fibrin clot and it is clear that they have arisen by a transformation of its elements and not from an intracellular action. The cells which are present stand out distinctly from the fibers, as shown in the figure, and show no connection with them.

In Fig. 8 is shown, at a magnification of 787 diameters, a longitudinal section of another wound which is practically the same age as the one shown in Figs. 6 and 7. In Fig. 8, however, we find that the formation of the new tissue has proceeded more rapidly and consequently the original fibrin net has been entirely transformed into the new fibrous tissue which fills the wound cavity. Blood vessels are forming in various regions in this new tissue and numerous cells, which for the most part show the typical spindle shape of fibroblast cells, are present but stand out clearly and distinctly from the fibers. The question of the relations existing between the new fibrous tissue and the cells which wander into it from the surrounding edges of the skin is one which has been studied with care. It has already been pointed out that the transformation of the fibrin clot occurs previous to the appearance of any cells, so that it cannot be due to an intracellular action (Figs. 3 and 4). A study of the preparations shows that the cells when they first appear in the new fibrous tissue are round in shape (Figs. 5 and 7). Later they elongate and assume the typical spindle shape of fibroblast cells (Fig. 8). This change in the shape is apparently due to the stereotropic action of the cells in moving along the fibers.<sup>6</sup> The movement of the cells in the newly formed fibrous

<sup>6</sup> Harrison, R. G., The Reaction of Embryonic Cells to Solid Structures, *Jour. Exper. Zool.*, 1914, xvii, 521.

tissue appears to result in a separation of the bundles of fibers and we have as a result what, to all appearances, is a typical scar tissue. With the Mallory stain the cells are colored yellow and they are distinctly differentiated from the fibers of the new tissue which are colored blue by the stain. The preparations do not show any evidence of a digestion by the cells of the new fibrous tissue or of any attempt by them to form new fibers intracellularly. The condition shown in Fig. 8 is typical of the condition found in much older wounds (3 to 4 weeks) and the evidence appears clear from these results that, in the healing of skin wounds in the frog, the new fibrous tissue present in the wound which has been formed by a direct transformation of the fibrin clot remains as the permanent connective tissue.

#### *Nature of the New Fibrous Tissue.*

The appearance, structure, and staining reaction with Mallory's stain of the new fibrous tissue are, as shown in the previous paper, identical with those of regularly formed connective tissues. In an endeavor to settle definitely the real relationship existing between regular frog connective tissue and the new fibrous tissue several series of tests have been made.

*Staining Reactions of the Fibrous Tissue Formed in Wounds.*—The Mallory connective tissue stain, either modified or unmodified, has proved to be more specific in its reaction than any other stain that has thus far been used in the work, although various other methods have been tried. This stain will color connective tissue fibers of the frog an almost perfect ultramarine blue if they are not too closely packed together. If, for example, a piece of skin from an adult frog is stained with Mallory's stain it will be found, in general, that the stratum spongiosum, in which the bundles of connective tissue fibers are loosely packed together and in which the individual fibrils can be distinguished, will be colored an ultramarine blue. On the other hand, in the stratum compactum of the same preparation in which the bundles of fibers are closely massed, it will be found that this layer will be stained a color varying from yellow to bright red, depending upon the manipulation of the stain.

The new fibrous tissue formed in the clots shows the same color re-



action to a Mallory stain as does the connective tissue in the skin. When the bundles of fibers in the new fibrous tissue are not closely matted together, as shown for example in Figs. 3, 4, and 5, the Mallory stain gives them a typical blue color as in the stratum spongiosum of the skin. On the other hand, when the new fibrous tissue is very compact and the individual fibrils composing the bundles cannot be distinguished, then, in many cases, the result will be a color varying from yellow to red, similar to that found in the stratum compactum of the skin. The same color also generally results from the Mallory stain in very young preparations in which the clot has not been changed into the new fibrous tissue but retains its original structure.

With a Van Gieson micro-fuchsin stain the new fibrous tissue shows a negative reaction. This stain, however, does not appear to be a specific one for connective tissues of the frog. The results show that it acts in just the opposite way that Mallory's stain does, in that it only gives the characteristic red color for connective tissues when a heavy tissue is present in which the bundles of fibers are closely massed together. In the frog skin, for example, the stratum compactum occasionally stains red, whereas in the stratum spongiosum an entirely negative reaction is obtained. In most cases, however, all the connective tissues of the frog skin as well as those from other parts of the body give a negative color reaction with this stain. In this connection a series of staining tests were made with embryonic connective tissue from the tail of a 50 mm. tadpole. It consists of a mass of connective tissue fibers loosely packed together. These fibers will take a typical stain with Mallory's stain, but with Van Gieson's the reaction is negative, as it is with the new fibrous tissue. The results in brief from all these experiments show that the Van Gieson's stain is not specific for connective tissue fibers in the frog whether embryonic or adult, and the same is true for its reaction with the new fibrous tissue.

*Digestion Tests of the Fibrous Tissue Formed in Wounds.*—Of all the tests used to distinguish between fibrin and connective tissues, the digestion tests are regarded as being the most specific and conclusive. A young fibrin clot is easily digested in a pancreatin solution whereas connective tissues resist the action. In the previous paper it was noted that the transformed fibrin net in the tissue cultures was easily dissolved in

pancreatin, and this fact gave evidence that the new fibrous tissue was fibrin in character although greatly changed in appearance from the typical fibrin net. However, it was also pointed out that in this case the test was not conclusive, inasmuch as the newly formed fibers in the clot were imbedded in a fibrin net which was not completely transformed, and the digestion of the unchanged fibrin clot by the pancreatin would naturally cause a breaking down and scattering of the fibers which had formed in it, and remained connected with it. In other words, the surrounding unchanged fibrin constituted the sole support of the fibers which had formed from a part of it.<sup>7</sup> This close relation existing between the fibrin clot and the new fibrous tissue also prevents the digestion tests being used in the present experiments to furnish a conclusive answer as to the real nature of the transformed fibrin net tissue in the clots formed in wound healing.

In this connection the results secured by a series of experiments in which the embryonic connective tissues of the tadpole were subjected to pancreatin digestion should be noted. In these experiments pieces of skin, taken from tadpoles ranging in length from 7 mm. to large tadpoles of about 70 mm. just before metamorphosis, were placed in pancreatin solutions of uniform strength<sup>8</sup> and digested over night at 38°C. In all these experiments a complete digestion of the tadpole skin occurred. This same result was obtained when the connective tissue from the tails of tadpoles of various ages was digested in pancreatin solutions. These young fibers, as has been noted, stain typically with Mallory's stain and are apparently fully formed. Nevertheless they can be completely digested in pancreatin in a few hours. These results with embryonic connective tissues are entirely different from those obtained when connective tissues from an adult frog are subjected to the same treatment, for the experiments show that the mature connective tissues in the skin of an adult frog are able to resist the action of the pancreatin and remain as a mass of tissue which, as noted above, could be used in the implantation experiments.

<sup>7</sup> Baitsell, G. A., The Origin and Structure of a Fibrous Tissue Which Appears in Living Cultures of Adult Frog Tissues, *Jour. Exper. Med.*, 1915, xxi, 473.

<sup>8</sup> The pancreatin extract used in all these digestion experiments was obtained by the well known Roberts' method. The author is indebted to Professor F. P. Underhill for his assistance in this matter.

These results demonstrate that the reaction of the embryonic connective tissues of the frog to pancreatin digestion is the opposite of that of adult connective tissue.

The results of both the staining and digestion tests, in brief, show that with these methods one is unable to differentiate between the new fibrous tissue, formed by a transformation of the fibrin clots, and embryonic connective tissues of the tadpole, and that the connective tissues of the adult, while reacting in the same way to the stains, can be differentiated from both the new fibrous tissue and embryonic connective tissue by their ability to resist the pancreatin digestion.

There is the possibility that the same resistance to pancreatin digestion which must develop in embryonic connective tissue as it becomes mature, as shown by the digestion tests on the mature and embryonic connective tissues, might also develop in time in the new fibrous tissue which has resulted through a transformation of the fibrin clot, for it is well known that old fibrin clots become highly resistant to various agents which will entirely dissolve younger clots. Mall<sup>9</sup> says: "Fresh fibrin is easily dissolved in dilute acid or digested in pancreatin. After fibrin becomes older, as in fibrinous deposits, the fibrils become very resistant and are not dissolved in boiling acetic acid, 20 per cent, in cold concentrated KOH, H<sub>2</sub>SO<sub>4</sub>, HCl, HNO<sub>3</sub>, or nitro-hydrochloric acid." What changes take place in these fibrinous deposits that cause such an increase in their resisting powers is apparently not known. From the present results one would naturally suggest that in such cases, in the course of time, a complete transformation of the fibrin clot into the new fibrous tissue had occurred and that this transformed tissue, when old enough, was able to resist the action of pancreatin and various other destructive agents.

This point has proven to be one which is difficult of solution. The fact that even the fully formed connective tissue of large tadpoles is unable to resist the pancreatin digestion makes it impossible to expect that one could ever keep tissue cultures alive long enough to secure a new fibrous tissue from the fibrin clot which would be able to

<sup>9</sup> Mall, F. P., *Reticulated Tissue, and Its Relation to the Connective Tissue Fibrils*, *Johns Hopkins Hosp. Rep.*, 1896, i, 182, 194.

resist the pancreatin digestion and thus show definitely that it could not be distinguished from the regular connective tissues in this regard. In wounds the problem is complicated by the presence of the cells which wander into them and through the new fibrous tissue soon after it has been formed from the fibrin clot. The preparations, as stated above, do not show any evidence of an attempt by these cells to form new fibers intracellularly, but, nevertheless, their presence in the wound for the long period of time which evidently is necessary for the new fibrous tissue to become resistant, makes the problem a difficult one.

#### DISCUSSION.

The experiments reported in the present paper demonstrate that in wounds made in the skin of adult frogs there occurs, as has previously been shown to be the case in living cultures of adult frog tissues, a direct transformation of the fibrin clot into a new fibrous tissue without any intracellular action. This newly formed fibrous tissue which fills the wound space is apparently identical in appearance, structure, function, and staining reactions with regularly formed permanent connective tissue. It differs from adult connective tissue in the skin of the frog in its reaction with pancreatin digestion. However, this test, as well as all others that have so far been tried, fails to differentiate between the new fibrous tissue and young connective tissue found in tadpoles of various stages.

Two views might be held with reference to the fate of this new fibrous tissue directly formed from the fibrin clot. The first is that it is only a temporary tissue and that it will be replaced in time by a permanent connective tissue formed through an intracellular action of the fibroblasts. This is in accord with the generally prevailing views<sup>10</sup> held at present, which are that the fibrin, in wounds healing by first intention, forms a temporary tissue which holds the wound surfaces in place and stops up the wound thus preventing infection. Later the fibrin disappears, not by liquefaction, but through an actual consumption by the new tissue cells which show a positive chemotaxis to the fibrin mass and are thus attracted into the fibrin clot

<sup>10</sup> For a general discussion and summary see Marchand, F., *Der Process der Wundheilung*, Stuttgart, 1901, 52-55.

in large numbers. The permanent tissues which go to fill the wound space are then formed by these cells through an intracellular action.

The other view is that this new fibrous tissue remains in the wound as a permanent connective tissue. That is to say, at least in wound healing in the frog skin, a reaction occurs which is able to transform a fibrin clot by a direct intercellular action into a permanent fibrous connective tissue. This view is supported by the work of Hertzler<sup>11</sup> who finds that, in experimental peritoneal adhesions in the dog, a new fibrous tissue, which he believes remains permanently, is formed by a transformation of a fibrin net. Speaking of the formation of this fibrous tissue, he says:<sup>12</sup> "My researches have convinced me that the cell is not primarily the active agent, but that the initial processes are chemical and are identical with those of blood coagulation, the cell playing an entirely secondary rôle."

The results obtained in the present experiments also give evidence in favor of the latter view. The fibroblast cells which wander into the new fibrous tissue apparently do not digest the fibers which have previously been formed, nor is there any evidence revealed, by a thorough study of the preparations, of an attempt by these cells to form new fibers intracellularly. Their only apparent action is to break up the larger bundles of fibers by their movements among them. In a wound of about 12 days a typical scar tissue is generally present (Fig. 8), and later stages (3 to 4 weeks) do not show any further changes in the structure of the connective tissue present in the wound.

#### SUMMARY.

1. In experimental wounds, made by removing various sized pieces of skin from the frog, there is a rapid coagulation of the blood plasma and lymph to form a coagulation tissue which fills the wound cavity.
2. The observations on the living animals show that the coagulation

<sup>11</sup> Hertzler, A. E., Pseudoperitoneum, Varicosity of the Peritoneum and Sclerosis of the Mesentery. With a Preliminary Note on the Development of Fibrous Tissue, *Jour. Am. Med. Assn.*, 1910, liv, 351; The Development of Fibrous Tissues in Peritoneal Adhesions, *Anat. Rec.*, 1915, ix, 83.

<sup>12</sup> Hertzler, A. E., Pseudoperitoneum, Varicosity of the Peritoneum and Sclerosis of the Mesentery. With a Preliminary Note on the Development of Fibrous Tissue, *Jour. Am. Med. Assn.*, 1910, liv, 352.

tissue becomes more and more resistant and is generally of sufficient strength to hold the cut edges of the wound in place and to retain its position in the wound cavity. It serves, at least temporarily, as a connective tissue and as a base for the epithelial cells which rapidly move in from all the cut edges and cover the wound.

3. The study of the prepared sections of wound tissue show that at first in the coagulation tissue, formed as a result of the clotting of blood and lymph, a typical fibrin net is present in the wound (Fig. 3). Later this fibrin net is transformed into a new fibrous tissue containing bundles of wavy fibers in which, in many instances, the individual fibrils can be noted (Fig. 4). This transformation of the clot and the formation of the new fibrous tissue takes place before the tissue cells wander into the coagulation tissue and therefore cannot be due to an intracellular action. It is a direct transformation of the fibrin clot and is identical with the process which was previously found to take place in the fibrin clots in living cultures of adult frog tissues.

4. The tissue cells, which later move into the new fibrous tissue in large numbers from the surrounding areas, do not digest the fibers but, apparently by their movements, cause a division of the large bundles into smaller ones (Fig. 5). These cells when they first appear in the fibrous tissue are rounded, but later they assume the typical elongated spindle shape of fibroblast cells (Fig. 8). The preparations do not show any connection between these spindle-shaped cells and the fibers which had already formed, nor is there any evidence of a later attempt by them to form new fibers intracellularly.

5. The staining reactions of the new fibrous tissue appear to be identical with the staining reactions of the connective tissue in frog skin. However, the new tissue can be digested in pancreatin and in this reaction it differs from the connective tissue in the skin of the adult frog. On the other hand, extensive experiments with pancreatin on embryonic but fully formed connective tissue, obtained from the tail and skin of tadpoles of various ages, show that pancreatin will digest it just as it does the newly formed fibrous tissue.

## EXPLANATION OF PLATES.

All the figures, with the exception of Fig. 1, were drawn from prepared material which had been preserved in Zenker's solution, sectioned at  $10\ \mu$ , and stained with Mallory's connective tissue stain.

## PLATE 106.

FIG. 1. 3 day wound from back of frog, shown *in toto* from the under surface.  $\times 15$ . A piece of skin measuring about 5 by 2 mm. was removed and the cavity thus formed is shown filled with the coagulation tissue (C.T.). A considerable area of the skin tissue (SK.T.) surrounding the wound is shown, and blood vessels (B.V.) are also present.

FIG. 2. Transverse section of the preparation shown in Fig. 1.  $\times 77$ . Cut edges of the skin tissue (SK.T.) marking boundaries of the wound are shown at both ends of the figure. These are connected by the coagulation tissue (C.T.) in which fibers (FIB.) can be noted at various places. Epithelial cells (EP.C.) which have moved in from the surrounding areas overlie the coagulation tissue. Section of blood vessel (B.V.) and numerous blood corpuscles (B.C.) are also shown.

## PLATE 107.

FIG. 3. Transverse section showing a portion of the coagulation tissue from the same preparation as Figs. 1 and 2.  $\times 787$ . The figure shows the structure of the coagulation tissue present in a 3 day wound. Some of this tissue retains the fibrin net (F.N.) structure of the original fibrin clot. Other areas show new fibrous tissue (N.F.T.) which has been formed by a transformation of the elements of the fibrin net. Numerous blood corpuscles (B.C.) are present, but no fibroblast cells.

FIG. 4. Longitudinal section of a 4 day wound in the leg of a frog.  $\times 787$ . The coagulation tissue has been transformed into the new fibrous tissue (N.F.T.) in which bundles of wavy fibers can be seen. Transformation is most complete in the denser region of coagulation tissue lying to the right of the figure. No fibroblast cells are present, but a few blood corpuscles (B.C.) are shown which are evidently undergoing degeneration.

FIG. 5. Longitudinal section from the preparation shown in Fig. 4.  $\times 787$ . The section from which this drawing was taken was taken at a deeper level of the wound than Fig. 4; *i.e.*, just above the underlying muscle tissue. To the right the new fibrous tissue (N.F.T.) is more dense, but even here the fibrous structure can be seen. To the left it is broken up into wavy fibrous bundles among which numerous cells (FBL.C.) are appearing. Blood corpuscles (B.C.) are also present.

## PLATE 108.

FIG. 6. Transverse section of a 12 to 13 day wound preparation.  $\times 240$ . Cut ends of the skin tissue (SK.T.) are shown at the right and left of the figure.

The wound cavity is almost filled with the new fibrous tissue (N.F.T.) except for a small portion of fibrin clot (F.C.) lying to the right. Epithelial cells (EP.C.) are piled up many layers thick above the new tissue. Cells (FBL.C.) are also present in the new tissue. Compare Fig. 7.

FIG. 7. A portion of the preparation shown in Fig. 6.  $\times 787$ . This drawing shows the remains of a fibrin clot (F.C.) lying to the right in Fig. 6. It has lost its original fibrin net structure and has become a more or less compact mass. Numerous fibers can be seen running through it, and around the edges are a great many fibers which have resulted from a transformation of the fibrin clot and which go to form the new fibrous tissue (N.F.T.). Numerous rounded cells (FBL.C.) are present, some of which are beginning to assume the typical spindle shape of fibroblast cells. They stand out clearly and show no connection with the fibers.

#### PLATE 109.

FIG. 8. Longitudinal section of another 12 to 13 day wound preparation.  $\times 787$ . This figure shows complete transformation of coagulation tissue into new fibrous tissue (N.F.T.) which fills the wound cavity between the cut edges of the skin tissue (SK.T.). Blood vessels (B.V.) are forming and numerous spindle-shaped fibroblast cells (FBL.C.) are present. These cells stretch out along the fibers which had previously been formed through a transformation of the fibrin net and apparently separate the bundles. They do not digest the fibers nor is there any evidence of an attempt by them to form new fibers intracellularly.



B.V.      SK.T.      O.T.

FIG. 1.

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SK.T.

FIG. 2.

(Baltzell: Fibrous Tissue Formed in Wound Healing.)







FIG. 6.

FBL.C.

F.O.

FIG. 7.

(Baltzell Fibrous Tissue Formed in Wound Healing.)



5K.T.

FIG. 8.

(Baitsell: Fibrous Tissue Formed in Wound Healing )





## THE ABSORPTION OF ADRENALIN AFTER INTRA-TRACHEAL INJECTION.\*

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In order to subject a living organism to the systemic action of any soluble substance, it is obvious that the substance must first reach the circulating fluids of this organism; from the lymph and blood streams the drug then may pass into the tissues and exert its effect. The main routes available for bringing any substance into contact with the tissues are as follows: (1) by introduction into the gastro-intestinal canal; (2) by subcutaneous, intramuscular, intravenous, or intraspinal injection; (3) by inunction through the skin; and (4) through the respiratory tract.

Of these routes the first two are most frequently employed in human therapeutics and in the laboratory. Inunction is used only exceptionally at the present time, and practically the only drug administered through the skin for its systemic action is mercury.

The respiratory route also is not utilized to any extent, except for purely local effects, when it is desired to incorporate a drug. Syphilis was occasionally treated by allowing the patient to inhale the sublimated metal, but this method was never extensively employed because of the impossibility of judging the dose. Local affections of the respiratory passages, for example, laryngitis, bronchitis, and bronchiectasis, are treated by allowing the patient to inhale the vapors of boiling water to which various substances (creosote, eucalyptol, opium preparations, etc.) have been added. Experimentally the respiratory passages are practically not utilized when a drug is to be administered for its general action.

\* A preliminary report was published in the Proceedings of the Pharmacological Society (*Jour. Pharm. and Exper. Therap.*, 1914-15, vi, 608).

Among the relatively few experimenters who have used the intratracheal method for this purpose we may mention Külbs,<sup>1</sup> who injected rabbits repeatedly for a number of days with adrenalin, introducing the hypodermic needle into the trachea through the skin of the neck. He observed cough after the injection, and twice an animal died a few minutes after the injection, from pulmonary edema. In those animals which survived the injections, which were given every day or every other day for 22 to 78 days, the total amount being 3.4 to 14 cc. of adrenalin, he found the same macroscopic and microscopic alterations, though of smaller extent, which he obtained after intravenous injection of adrenalin.

Ephraim<sup>2</sup> in a series of papers reports the results of endobronchial treatment of chronic bronchitis and asthma in the human subject. Various drugs, including adrenalin, were introduced as a fine spray into the bronchi, often through a bronchoscope. The effects obtained were chiefly local; even after the bronchial administration of 1 mg. of adrenalin by means of a nebulizer, he obtained no definite rise of blood pressure. In a dog, however, the same method yielded a powerful rise of blood pressure when a large amount, 2 mg., was used.

The failure of Ephraim to obtain a rise of pressure after the administration of 1 mg. of adrenalin endobronchially in the human subject is perhaps attributable to the method. Ephraim himself observed in rabbits that the spray of a colored solution from a nebulizer, even when introduced into the trachea, did not reach the bronchi, the vapor being precipitated near the point of application. In the same way the endobronchial spray of adrenalin was possibly precipitated in the larger bronchi so that only negligible amounts reached the alveolar region where, as we shall show, the absorption is best.

That even colloids can be absorbed with rapidity when injected intratracheally is shown by Ishioka.<sup>3</sup> Ishioka sensitized guinea pigs by the subcutaneous injection of human serum and after 12 to 15 days injected 0.05 to 0.1 cc. of human serum into the trachea of these animals with the object of producing an anaphylactic pneumonia. He observed the milder anaphylactic symptoms in the majority of his experiments, but in two instances acute death with the typical lung picture resulted.

The absorptive capacity of the supratracheal respiratory passages does not concern us here; references concerning the effect of inhaled substances will be found in the papers by Ephraim.

Our own experiments will show that the intratracheal injections of adrenalin are rapidly absorbed even under disadvantageous conditions and exert a systemic effect, and also that this method may be of value therapeutically when a rapid action on the heart is desired.

<sup>1</sup> Külbs, *Arch. f. exper. Path. u. Pharm.*, 1905, liii, 150.

<sup>2</sup> Ephraim, A., *Deutsch. med. Wchnschr.*, 1911, xxxvii, 2079; 1912, xxxviii, 1453.

<sup>3</sup> Ishioka, S., *Deutsch. Arch. f. klin. Med.*, 1912, cvii, 500.

*Methods.*

The research was carried out entirely with rabbits, and the chief test substance employed was adrenalin. Adrenalin was chosen because its absorption could be readily detected by recording the blood pressure, and the character and degree of the resultant rise would give some indication of the speed and amount of absorption.

The animals were anesthetized by ether for the operative interferences. These were the insertion of a cannula into the right carotid artery; transection of the trachea with introduction and ligation of a wide glass tube about 2 cm. long into the distal stump so that the respiratory path was free and injection into the trachea easy; in some rabbits the tracheal cannula was inserted as near the thorax as possible and the lower end of the proximal trachea then ligated, thus converting the cervical trachea into a sac from which the absorption of adrenalin could be tested; in a number of experiments both vagi were sectioned in the neck.

The blood pressure was written by a mercury manometer connected with the artery by tubing filled with a half saturated solution of sodium sulphate.

The adrenalin used was generally the commercial solution in 0.1 per cent strength, preserved with chloretone. In some experiments, however, a solution was made from the commercial powder preparation, and this was used with or without the addition of chloretone.

The dose injected varied from 0.15 to 0.03 cc. per kilo of body weight. The maximum dose quoted was occasionally greatly exceeded when adrenalin was injected intramuscularly.

The solutions were injected into the respiratory passages in several ways. Usually the required amount was injected in 4 to 6 seconds from a Record tuberculin syringe into the tracheal cannula. In a number of experiments a filiform catheter was introduced into a bronchus and the solution then driven in by a gentle blast of air. A few times the solution was injected directly into the lung tissue by passing the hypodermic needle through the walls of the chest.

In a majority of the experiments doses of adrenalin were injected repeatedly, not only into the trachea but also into the erector spinæ muscles of the back.

The rabbits were always placed on an electric warming pad to reduce or prevent the loss of body heat.

**EXPERIMENTAL RESULTS.**

Twenty-three experiments were carried out in the adrenalin series; in ten of these tests both vagi were cut previous to the injection of the drug. The course of the experiments and the characteristic effects will be illustrated by a few typical protocols arranged in tabular form.

All rabbits tabulated were tracheotomized under light ether anesthesia; there was no insufflation of air except in No. 3 (Table I); vagi

were intact, except in No. 8 (Table IV); all injections into the trachea were made with a syringe through the tracheal tube.

TABLE I.

No. of animal.	Color. Sex. Weight.	No. of injection.	Method and site of injection.	Amount of adrenalin per kilo.	Latent period of blood pressure rise.	Blood pressure rise.	Duration of blood pressure rise.	Interval between injections.
				cc.	sec.	mm.	min.	min.
3	Gray ♀	1	Catheter into bronchus.	0.23	12	64 (104-168)	8	
		2	Trachea.	0.23	—	45 (90-135)	9	9
		3	"	0.23	6	25 (107-132)	9	10
		4	Erector spinæ muscle.	0.23	No rise in 4 min.			10
		5	Erector spinæ muscle.	0.46	" " " 4 "			4
		6	Trachea.	0.23	7	24 (96-120)	18	4
Vagi intact.	2,210 gm.							

Killed by medullary puncture after experiment. Lungs showed only a moderate degree of pulmonary edema.

6	Gray ♂	1	Tracheal sac.	0.25	52	16 (91-107)	15	
		2	Trachea.	0.25	12	60 (100-160)	—	17
		3	Tracheal sac.	0.25	No rise in 6 min.			16
		4	Trachea.	0.25	3	40 (62-102)	10	8
		5	Erector spinæ muscle.	0.5	No rise in 7 min.			10
		6	Trachea.	0.25	4	26 (80-106)	7	10
Vagi intact.	2,100 gm.							

Killed later by medullary puncture. The lower lobes of both lungs showed well marked pulmonary edema; slight in upper lobes.

TABLE II.

No. of animal.	Color. Sex. Weight.	No. of injection.	Method and site of injection.	Amount of adrenalin per kilo.	Latent period of blood pressure rise.	Blood pressure rise.	Duration of blood pressure rise.	Interval between injections.
				cc.	sec.	mm.	min.	min.
7	Gray ♂ 1,610 gm.  Vagi intact.	1	Trachea.	0.12	2	50 (100-150)	6	
		2	"	0.12	4	39 (88-127)	12	9
		3	Erector spinæ muscle (left).	0.6	3	43 (99-142)	20	13
		4	Trachea.	0.12	8	21 (98-119)	10	21
		5	"	0.12	5	25 (95-120)	12	15
		6	Erector spinæ muscle (right).	0.28	No rise in 8 min.			14
		7	Trachea.	0.12	3	24 (92-116)	21	8
		8	"	0.24	10	10 (102-112)	5	20

Killed later by medullary puncture. Lungs collapsed well and showed only a moderate degree of pulmonary edema.

8	White ♀ 2,365 gm.  Vagi cut.	1	Trachea.	0.21	6	64 (114-178)	15	
		2	"	0.21	10	30 (110-140)	6	18
		3	"	0.21	8	16 (116-132)	9	8
		4	Erector spinæ muscle.	0.42	No rise in 4 min.			15

Pink fluid poured from trachea 13 min. after first dose. About 5 min. after last injection, blood pressure fell abruptly, convulsions, death. Lungs showed marked pulmonary edema.

TABLE III.

No. of animal.	Color. Sex. Weight.	No. of injection.	Method and site of injection.	Amount of adrenalin per kilo.	Latent period of blood pressure rise.	Blood pressure rise.	Duration of blood pressure rise.	Interval between injections.
4	Gray 2,160 gm.	1	Erector spinæ muscle (left).	0.23	14	34 (106-140)	4	
		2	Jugular vein.	0.23	At once.	43 (107-150)	10	9
		3	Trachea.	0.23	8	20 (110-130)	10	9
		4	Erector spinæ muscle (right).	0.23	No rise in 2 min.			11
		5	Trachea.	0.23	10	10 (100-110)	3	2
		6	Erector spinæ muscles.	0.23	No rise.			

After last injection foam and pink fluid poured from trachea (marked pulmonary edema).

A study of these typical protocols as given in Tables I, II, and III shows with clearness all the points we wish to emphasize, and they will now be considered in detail.

*Latent Period.*—The latent period elapsing between injection and the onset of the blood pressure rise varied between 2 and 38 seconds, but usually was less than 10 seconds; the general average of all first injections was 13 seconds. Repeated injections exerted no uniform effect on the length of the latent period. Thus in Experiment 7 the first dose of 0.12 cc. of adrenalin per kilo injected into the trachea surely reached a physiologically effective concentration in the blood within 2 seconds, for after that interval the blood pressure began to rise. The seventh injection of the same dose in the same place, 80 minutes later, exerted a blood pressure effect after a latent period of only 3 seconds, in spite of the fact that the animal had received intratracheally and intramuscularly in the interval between the two injections mentioned 1.24 cc. of adrenalin per kilo, divided into five doses. A still more striking example of the speed of absorption is

given in Experiment 8. In this experiment the vagi had been cut before administering the adrenalin, and the first dose of 0.21 cc. per kilo into the trachea caused a pulmonary edema of such an extent that foam and pinkish fluid poured from the tracheal cannula. Nevertheless, a repetition of the same dose in the same place a few minutes later exerted a good blood pressure effect after a latent period of only 10 seconds. In this instance the area available for absorption was undoubtedly reduced considerably by the pulmonary edema, yet the speed of absorption was still rapid. All the tabulated protocols show a similar rapid absorption after intratracheal injections.

The latent period or speed of absorption was not apparently affected by the amount of adrenalin injected; for example, in Experiment 3 the intrabronchial injection of 0.23 cc. per kilo by means of a catheter showed a latent period of 12 seconds before the blood pressure began to rise. In Experiment 7, on the other hand, a much smaller dose per kilo, 0.12 cc., injected into the trachea gave a blood pressure effect after a latent period of only 2 seconds.

*Quantitative Absorption.*—The quantitative absorption cannot be definitely established, yet the blood pressure rises obtained after the various injections furnish some indication of the amount of adrenalin absorbed. Examination of the absolute values of the blood pressure rises in mm. of mercury obtained after successive injections of the same dose of adrenalin in the same place, the lung passages for example, shows that the blood pressure effect in general decreases with the number of injections. Thus in Experiment 7 five doses of 0.12 cc. of adrenalin per kilo were injected into the trachea, the time intervals varying between 9 and 34 minutes if the interpolated intramuscular injections are included. The blood pressure rises after these five tracheal injections were 50, 39, 21, 25, and 24 mm. In Experiment 8 the successive decrease of effect is more pronounced. In this experiment 0.21 cc. of adrenalin per kilo into the trachea at intervals of 8 to 18 minutes gave the following rises of blood pressure: 64, 30, and 16 mm. In this animal, however, the vagi had been sectioned previous to the first injection of adrenalin, and after the first dose a marked pulmonary edema developed during which fluid poured from the tracheal cannula. The presence of so much fluid decreased the absorptive area available, and in addition diluted the adrenalin with a colloidal

solution, rendering absorption still slower, yet in spite of this the speed of absorption was not appreciably delayed, the latent period being 10 and 8 seconds, but the amount absorbed, as judged by the pressure rises, showed practically 50 per cent decreases.

This decrease in effectiveness in causing a blood pressure rise shown by successive tracheal injections is in accord with the observations of Meltzer and Auer<sup>4</sup> that adrenalin diminishes absorption from the tissues. These authors demonstrated, among other facts, that the intravenous injection of adrenalin delays the absorption of strychnine or fluorescein from the subcutaneous tissue, and also that repeated intramuscular injections give a decreasing blood pressure effect, due to diminished absorption.

It will be noticed on examining the tables that all injections of adrenalin into the trachea gave some blood pressure effect; in other words, that some absorption took place even under unfavorable circumstances. A comparison was therefore made between the absorptive capacity of the lung and that of the erector spinæ muscles. The erector spinæ muscle was chosen because this thick mass of muscle is composed of fine fibers not separated into coarse fasciculi, like the glutei for example, and in addition is surrounded by a dense fascia which exerts pressure upon the injected substance, thus facilitating absorption. Injection into this muscle has been shown by Meltzer and Auer<sup>4</sup> to be practically equivalent to an intravenous injection.

The experimental test gave important results. It clearly appeared that the injection of several doses of adrenalin into the lung passages reduced the absorption from the erector spinæ muscles to such a degree that even double the intratracheal dose given intramuscularly did not enter the circulation in sufficient amount to affect the blood pressure; nevertheless, another injection of the original dose of adrenalin into the lungs promptly entered the circulation in sufficient concentration and amount to cause a blood pressure rise (see Experiments 3, 6, 7, and 4). This fact appears with especial clearness in Experiment 3. In this test the rabbit received three doses each of

<sup>4</sup> Meltzer, S. J., and Auer, J., *Tr. Assn. Am. Phys.*, 1904, xix, 207; *Jour. Exper. Med.*, 1905, vii, 59. Auer and Meltzer, *ibid.*, 1911, xiii, 328.



0.23 cc. of adrenalin per kilo into the lung, the time consumed being about 29 minutes. Each injection gave a rise of blood pressure (64, 45, and 25 mm.). Then the same dose was injected intramuscularly, but no blood pressure rise occurred in 4 minutes. The dose was then doubled and 0.46 cc. per kilo was injected into the other erector spinæ muscle, but again no blood pressure rise followed. 4 minutes later the original dose of 0.23 cc. was injected into the lung passages and after a latent period of 7 seconds the blood pressure began to rise and reached an absolute value of 24 mm. of mercury.

The failure to obtain a blood pressure effect from an intramuscular injection of adrenalin under the conditions mentioned is, however, not absolute. If the dose administered intramuscularly is increased sufficiently, enough adrenalin will be absorbed to cause a blood pressure rise. This is illustrated in Experiment 7. Two lung injections of 0.12 cc. of adrenalin each had been injected in about 22 minutes. Then five times the dose (0.6 cc. per kilo) was injected intramuscularly; after a latent period of 3 seconds the blood pressure rose 43 mm. That there was, nevertheless, a definitely diminished absorption from this large intramuscular injection is shown by the fact that the rise of blood pressure, 43 mm., is even less than that caused by the first lung injection of only one-fifth the dose, which latter raised the pressure 50 mm. Subsequent injections of adrenalin into the lungs all gave rises of blood pressure, but an interpolated intramuscular injection of double the pulmonary dose gave no blood pressure effect. The amount absorbed from the muscles had fallen below the level of a physiologically effective dose.

In some experiments the conditions were still further varied by preceding the lung injections of adrenalin by intramuscular and intravenous injections of the same substance. Experiment 4 is one of this type. The adrenalin dose was always 0.23 cc. per kilo. After an intramuscular and an intravenous injection, absorption of adrenalin from the lung was by no means prevented, the blood pressure rising 20 mm. after a latent period of 8 seconds. A subsequent intramuscular dose, however, produced no blood pressure rise within 2 minutes. Another lung injection even now caused a definite rise of pressure.

These experiments definitely show that, under the conditions

mentioned, absorption from the lungs occurs with doses of adrenalin which are ineffective when injected intramuscularly.

There was no definite relationship to be observed between the amounts of adrenalin injected into the lungs and the resultant blood pressure rise. The same dose per kilo often produced widely different rises in different animals, and smaller doses often caused greater elevations of pressure than larger ones. Table IV illustrates this and demonstrates in addition that the amount of adrenalin injected played no part.

TABLE IV.

No.		gm.			mm.
5	Vagi intact.	1,650	0.3 cc. per kilo (0.5 cc.)	4 sec. latent period.	33 rise (107-140)
" 7	" "	1,610	0.12 cc. per kilo (0.2 cc.)	2 " " "	50 rise (100-150)
" 8	" cut.	1,980	0.25 cc. per kilo (0.5 cc.)	18 " " "	30 rise (114-144)
" 13	" "	1,790	0.25 cc. per kilo (0.4 cc.)	18 " " "	82 rise (116-198)

*Blood Pressure.*—The duration of the blood pressure elevation was usually less than 10 minutes; if only the effects of the first tracheal injections are considered the average is 6 minutes. Subsequent injections, however, often showed a definitely longer duration of the elevation. An illustration of this effect will be found in Experiment 7.

The character of the rise varied somewhat; it was usually more or less abrupt, the maximum being reached within 30 seconds. In other instances the maximum elevation was reached in about 1 minute. The abruptness of the rise seemed to bear some relation to the number of preceding injections, the slope becoming less steep with succeeding doses. This, however, was by no means true of all experiments, for in some all intratracheal injections gave sharp rises of pressure. Vagus pulses were often observed, but their occurrence was not as frequent as when the adrenalin is administered intravenously or intramuscularly.

In a number of experiments abrupt and profound drops of blood pressure were noted. These occurred without any warning during the maximum pressure elevation, the pressure falling within a few

seconds to 20 mm. and even less. These drops lasted from 30 to 160 seconds, and were not necessarily fatal. In some instances a number of these profound drops occurred, recovery of the blood pressure taking place spontaneously after a series of convulsions. When these drops occurred, they were always associated with more or less pronounced signs of pulmonary edema. The significance of this phenomenon will be discussed in a later paper.

*Site of Absorption.*—In order to obtain some information regarding the site of absorption of the injected drug, a few experiments were made with intratracheal injections of India ink or suspensions of lampblack in an extremely dilute gum arabic solution. Doses of 0.3 cc. per kilo of body weight were injected slowly by syringe into the tracheal cannula. 10 seconds after the injection the medulla was destroyed by puncture, and the lungs and trachea were immediately excised and examined. The three experiments carried out gave concordant evidence: the posterior and diaphragmatic surfaces of the lower left lobe always showed a large number of discrete and confluent, irregular black spots varying from about 2 to 5 mm. in diameter. The surfaces of the other lobes showed only a few or no spots, and these were confined largely to the posterior surfaces near the hilus of the right lower and right middle lobes.

On sectioning the lungs through the trachea and bronchi, the larger part of the left lower lobe was found to be a black mass containing foam and some fluid. The right lower and right middle lobes near the hilus also contained an amount of pigment which was greater than would be expected from the surface indications. The upper lobes and the median lappet showed a few spots in the body of these divisions.

The sections of the lung containing the pigment were larger and fuller than those free from it; moreover, they contained more fluid. The distension was greater than the amount of fluid present explained, and apparently was at least partly caused by a mechanical plugging of the bronchioles and infundibular ducts by the pigment. The amount of fluid present in the tissues seemed greater than the amount injected (0.3 cc.), so that perhaps some degree of pulmonary edema also developed.

These experiments with the tracheal injection of pigment suspen-

sions thus indicate that a certain amount penetrates to the alveoli, chiefly of the left lower lobe, within less than 1 minute, and that absorption in all likelihood takes place there.

It might be thought that some absorption could take place from the mucosa of the trachea and the bronchi, and such absorption indeed does take place, at least as far as the tracheal mucosa is concerned. This absorption from the tracheal mucosa is, however, quite slow and the blood pressure rise obtained sets in very slowly. We tested the absorptive power of the tracheal mucosa in the following way. The tracheal cannula was inserted as low as possible in the neck; the upper section of the trachea was then ligated near the cannula, converting it into a sac. Injections of adrenalin were then made into this sac and the blood pressure effect was noted. In the two experiments made the first injection of adrenalin gave each time a slow and gradual rise of pressure, the latent period being respectively 150 and 52 seconds. In the first experiment the blood pressure rise equaled 59 mm. and lasted longer than 15 minutes. In the second experiment (Experiment 6) the first injection into the tracheal sac gave a rise of only 16 mm. of mercury after a latent period of 52 seconds, the rise persisting for more than 15 minutes. A subsequent repetition of the injection gave no blood pressure effect within 6 minutes. The doses of adrenalin injected were respectively 0.3 and 0.25 cc. per kilo.

These experiments show that while absorption of adrenalin does take place from the tracheal mucosa, and therefore probably also from the bronchi and bronchioles, these surfaces play only a subsidiary part as sites of absorption when adrenalin is injected into the trachea.

Section of the vagi in the neck before the intratracheal injection of adrenalin yielded interesting results. These nerves were cut in order to prevent the occurrence of those profound blood pressure drops mentioned previously, on the assumption that they were due to the well known initial effect of adrenalin upon the vagus center. However, these drops still occurred in some of the experiments after section of the vagi, and in addition, pulmonary edema and sudden death were much more frequent than in the series with vagi intact. One intratracheal injection of adrenalin often sufficed to bring on a strong pulmonary edema and even death within a few

minutes. Without entering here into a discussion of all the phenomena observed, it may be said, in general, that section of the vagi produced no noteworthy alteration in the absorptive power of the lung tissue as far as adrenalin is concerned. The variations observed in the vagotomized series fell well within the range of those seen in the normal series, though a percentage reckoning of all intratracheal injections given for the first time, shows that the vagotomized animal exhibited a shorter latent period (10 against 13 seconds), a higher blood pressure rise (56 against 46 mm.), and a longer duration of the pressure elevation (9.5 against 6 minutes). Not much stress, however, should be laid upon averages gained from only twenty-three experiments, especially in this work where pulmonary edema entered as a complicating factor in the vagotomy series.

A number of experiments were also carried out with a 0.1 per cent solution of adrenalin made from the commercial powder, a few drops of concentrated hydrochloric acid being added to the sterile saline to bring about solution. This solution, with or without the addition of chloretone, did not give as good results when injected intratracheally as the solution obtained in the open market; the absorption was slower and the resultant rise of blood pressure less marked.

A few experiments were also carried out with the sodium salt of fluorescein. Solutions of this substance, in 1 or 10 per cent strength, were injected intratracheally, the dose being 0.3 cc. per kilo of body weight. Samples of blood were then taken at regular intervals from the carotid artery, allowed to clot in small test-tubes, and the serum was examined for fluorescence. These experiments also indicated a rapid absorption though not as striking as with adrenalin; after 15 to 30 seconds the blood samples showed the first detectable green fluorescence. This fluorescence rapidly increased at first, then more slowly, reaching a maximum after a number of minutes.

#### DISCUSSION AND SUMMARY.

In the preceding pages we have submitted evidence which shows that a simple intratracheal injection of a solution in a normally breathing rabbit penetrates within a few seconds to the alveoli, chiefly those of the left lower lobe; that absorption is rapid and well maintained;

and that the procedure may be repeated effectively a number of times even with a substance like adrenalin which decreases absorption. It was also shown that absorption of adrenalin from the lung could be obtained at a time when double the dose given intramuscularly exerted no blood pressure effect whatever, and that absorption could still take place after the development of pulmonary edema, when there was an undoubted dilution of the injected solution with a serum-containing liquid and when a diminution of the absorptive field had occurred.

The solution injected, after reaching the alveoli, is probably largely taken up by the capillaries of the pulmonary veins. This is indicated by the great rapidity with which an intratracheal injection of adrenalin may cause a rise of blood pressure. In numerous instances, for example, the pressure began to rise less than 5 seconds after the completion of an injection, equaling and even surpassing in rapidity of effect an intramuscular injection. Absorption by the lymphatics probably plays a secondary part, an assumption rendered all the more likely if we consider that lymph nodes are interpolated in the lymphatic pulmonary path, where the bed of the lymph stream becomes greatly widened and the current slowed.

Injection into the lungs, however, offers another advantage due to the vascular arrangement of the absorbing field which could be of value therapeutically. Absorption of liquids injected into the lung probably takes place largely through the capillaries of the pulmonary veins; to a slight extent possibly through the capillaries of the bronchial veins which empty partly into the pulmonary veins, partly into the azygos veins; and probably some absorption occurs also through the lymphatics. By far the larger proportion of the absorbed material will thus be rapidly delivered to the left auricle and then to the left ventricle. At each succeeding systole, as long as absorption continues, a fraction of the drug will be driven into the coronary arteries and be able to affect the musculature of the cardiac pump. This fact ought to render the procedure of intratracheal injection a valuable method when it becomes imperative to stimulate a suddenly failing heart as promptly as possible by drugs of the digitalis group.

Intratracheal injection is perhaps better under the conditions mentioned than the intravenous route, for the surface veins cannot

always be entered with promptness and certainty even under fairly normal conditions, and in cases of cardiac weakness the difficulties will be measurably increased, while an intratracheal injection can be carried out with ease. Moreover, it is legitimate to expect that some absorption will take place from the lung alveoli as long as the heart-lung circulation persists, no matter how feebly, and that thus some of the drug will reach the heart to act on this structure itself more promptly perhaps than when the drug is administered successfully through surface veins. As far as the intramuscular route is concerned, we have shown that the intratracheal injection of adrenalin gives prompt though diminished absorption at a time when double the dose intramuscularly exerts no blood pressure effect whatever.

The technical difficulties of giving an intratracheal injection in animals are slight. Tracheotomy as practised by us in the present series of experiments is not necessary, for the injection may be given into the intact trachea without exposure of the trachea. The hypodermic needle is inserted through the skin about 1 cm. below the larynx in a slanting caudad direction; the entrance of the needle into the trachea is readily felt. The injection should not be so rapid that the injected solution fills the entire tracheal lumen, but it should flow down the sides of the trachea. If the lumen is entirely filled, an expiration may drive some of the injected liquid into the larynx causing cough. In our experiments each injection of about 0.5 cc. consumed approximately 5 seconds.

In the human subject no data are available as far as our knowledge goes, but *a priori* it would seem that an intratracheal injection is almost as simple as in the lower animals. The free hypodermic needle could be inserted into the tracheal lumen immediately below the cricoid cartilage. The needle itself should preferably be connected with the syringe by a short length of rubber tubing to minimize the danger of breaking the needle by a sudden move of the patient. The amount of the solution should not be too small, so that at least a fraction of it may reach the alveoli as promptly as possible; 3 to 5 cc. probably would suffice.

Insertion of the needle in the locality mentioned would puncture the isthmus of the thyroid, but this is of no significance, especially when the procedure is employed in cases of cardiac failure where the

gravity of the condition would warrant incurring much heavier risks than a slight bleeding from the thyroidal isthmus.

In conclusion it may be said that the incorporation of drugs by intratracheal injection, while not as generally applicable as other methods, nevertheless has advantages which warrant its use also in human therapeutics.



# THE PRODUCTION OF AMYLOID DISEASE AND CHRONIC NEPHRITIS IN RABBITS BY REPEATED INTRAVENOUS INJECTIONS OF LIVING COLON BACILLI.

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PLATES 110 to 115.

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The experiments here reported are a portion of a larger series in which several varieties of bacteria were used and which were undertaken in the hope of producing in rabbits by repeated intravenous injections of living bacteria, over long periods, kidney lesions which might at least approximate the chronic lesions seen in man, which are probably due to long continued septic conditions. The results in rabbits injected with *Bacillus coli communior* were so striking in the constant and pronounced production of amyloid in various organs, as well as in the resulting kidney lesions apart from the amyloid deposits, that a brief report on them and a review of previous work on the experimental production of amyloid seems appropriate.

Frisch (1) in 1877 was the first to announce the experimental production of an amyloid-like substance. He produced a keratitis in rabbits by injection into the cornea of fresh blood from a case of anthrax. He reports positive results in 4 out of 300 corneas injected. The resulting lesion gave characteristic reactions with iodine and with iodine and sulphuric acid, but the reaction with aniline dyes was lacking and the material was doubly refracting. It seems doubtful, therefore, whether the substance was amyloid.

Birch-Hirschfeld (2) observed amyloid in the spleen of a rabbit which died after 6 weeks of chronic subcutaneous suppuration produced by the injection of pus from a case of osteomyelitis of the tibia. Other similar experiments gave negative results.

Bouchard and Charrin (3) observed amyloid in two rabbits. One had been injected subcutaneously with *Bacillus pyocyaneus* and subsequently four times intravenously at intervals of several months. The animal lived about 1 year. Amyloid was found in the kidneys and also in the vessels of the heart and in the heart muscle. A second rabbit lived 34 days after injection with material from

a human tuberculous lesion and with a culture of tubercle bacilli. Amyloid was present in the kidneys; there was none in the spleen, liver, or heart.

Czerny (4) in his first paper reports the production of amyloid in the spleen and in a few vessels of the kidneys of two dogs in which he had produced subcutaneous suppuration for 10 and 13 weeks by the injection of turpentine. The material deposited gave the iodine-sulphuric and methyl violet reactions. Czerny's paper is concerned especially with the iodophilic granules of leukocytes from which he believes amyloid is formed. In a second paper (5) he reports the production of amyloid by the same method in three more dogs, in one after suppuration lasting  $7\frac{1}{2}$  months, and in two after 4 months.

Condorelli-Maugeri (6) in a brief communication simply states that he has produced amyloid disease in the liver and kidneys of rabbits by injection of *Bacterium termo*. As far as the writer can discover, no detailed account of his experiments has been published.

Krawkow (7, 8, 9, 10) was the first to study systematically the question in a large series of different animals. He produced chronic suppuration by the subcutaneous injection of broth cultures of *Staphylococcus aureus* into rabbits, dogs, hens, doves, and frogs. In eight of twelve rabbits he produced amyloid, mainly in the spleen, in certain instances also in the gastro-intestinal tract, liver, kidneys, and salivary glands. In dogs the results were negative after subcutaneous suppuration lasting 2 to 3 months. The results in pigeons were also negative. He found amyloid much easier of production and more extensive in hens than in other animals, three of four hens and each of four cocks giving positive results in  $1\frac{1}{2}$  to 2 months. In two frogs, of several dozen injected, the spleens showed traces of a substance giving a suggestive reaction, but Krawkow was not certain that it was amyloid.

Krawkow (9) also tried Czerny's method of injecting turpentine subcutaneously and also silver nitrate, both with negative results. He believes that the abscesses produced by Czerny were not aseptic. He also injected rabbits and dogs over long periods with filtrates of cultures of *Staphylococcus aureus* and also with non-filtered cultures killed at 100°C. with negative results. Amyloid was found, however, in one rabbit which in 2 months had received seventeen injections of 5 to 30 cc. each of filtrate of a broth culture of *Bacillus pyocyaneus* killed at 100°C. In this experiment amyloid was present in the spleen in the small arteries and to some extent about the capillaries. In the kidneys traces were found about the convoluted tubules, but none in the glomeruli. None was found in the liver. As pointed out by several later workers, Krawkow's results are surprising when one considers the length of time of his experiments. In two rabbits amyloid was found in the spleen 2 days after the first injection of 10 cc. of broth culture of *Staphylococcus aureus*. In a third amyloid was pronounced after 11 days, during which time two injections of 2 and 3 cc. had been given, while in other cases injected repeatedly over a period of months, it was entirely lacking.

Davidsohn (11) confirmed the observation of Krawkow of the possibility of producing amyloid by subcutaneous suppuration resulting from repeated in-

jections of *Staphylococcus aureus*. He used rabbits, mice, hens, guinea pigs, and cats. In guinea pigs and cats the results were negative; the best results were obtained with mice and rabbits. There is the same inconsistency in time and the amount of culture injected as in Krawkow's experiments. Amyloid was found in the spleen, stomach, intestines, kidneys, and rarely in the salivary glands, pancreas, and lymph nodes. In only one instance doubtful amyloid was found in the bone marrow. In the kidneys amyloid was present in patches in small arteries of the medulla and along the collecting tubules; there was no change in the glomeruli. He obtained negative results with streptococcus, *Bacillus coli*, and putrefactive bacteria. Davidsohn believes that Czerny's positive results with turpentine may have been due to bacterial contamination of the lesions.

Lubarsch (12) was at first unable to produce amyloid by Czerny's method. Later (13) he produced suppuration for 9 to 21 weeks in eight dogs by the injection of turpentine. In one case of 16 weeks' duration, he found amyloid in the spleen only. A second was doubtful, the others negative. Seven rabbits were injected repeatedly subcutaneously with living broth cultures of *Staphylococcus aureus*, the longest period being for 9½ weeks. Positive results were obtained in two cases, of 5½ and 7 weeks. Amyloid was found in the spleen only. Guinea pigs were injected with living *Staphylococcus aureus* and dead broth cultures of *Bacillus pyocyaneus*, pneumococcus, streptococcus, and Friedländer's bacillus with negative results. Lubarsch calls attention to the irregularity of results, especially as to time, of his own experiments and those of others, and concludes that no certain method of producing amyloid has been found.

In later papers Davidsohn (14, 15, 16) reviews his own work and that of others and reports the subcutaneous injection of mice with gonococcus with positive results in two cases. His experiments with bacterial toxins gave negative results. With dead bacteria, however, he obtained positive results in one mouse in which amyloid was found in the spleen, liver, kidneys, and intestines.

Nowak (17) attempted to prove and extend the work of Krawkow, and his results seem worthy of reviewing in some detail. He experimented on rabbits and hens and found it much easier to produce amyloid in the latter. He used staphylococcus, streptococcus, *Bacillus pyocyaneus*, *Bacillus coli communis*, and their sterile filtrates; bouillon inoculated with human stool and a filtrate of this; also tuberculin, fresh and sterile pus, croton oil, and turpentine. Of seven rabbits injected with living *Staphylococcus aureus*, two, of 10 and 102 days' duration, showed amyloid in the spleen only. All hens so injected showed amyloid. Two rabbits injected with living *Bacillus pyocyaneus* were negative; two hens were positive. Two rabbits injected with living *Bacillus coli* and also two hens showed no pus and no amyloid. Rabbits injected with putrefied bouillon gave negative results, while each of two hens were positive. Of the rabbits injected with sterile filtrates of cultures of various organisms, one rabbit only, which had been injected with a filtrate of a culture of *Bacillus pyocyaneus* for 60 days, showed a few nodular deposits in the spleen which gave the amyloid reaction with aniline colors. Of nine hens injected with sterile filtrates, three were positive; two of these had

been treated with filtrates of *Staphylococcus aureus* cultures, and one with a filtrate of cultures of *Bacillus coli*. Of two rabbits and two hens injected for long periods with large doses of tuberculin, one of each showed nodules in the spleen which gave a doubtful reaction. Nowak considers them negative. Of three rabbits injected with fresh pus, one, in 8 days, showed amyloid in the spleen and liver; of two hens similarly injected, one showed amyloid in the spleen and liver. Rabbits injected with sterile pus were negative; two hens were both positive. Croton oil gave negative results. Of two rabbits injected with turpentine, one showed amyloid in the spleen and liver in 201 days; each of five hens so injected showed amyloid in the spleen and liver, and two of these in the kidneys also.

Maximow (18) also obtained positive results in hens and rabbits by repeated subcutaneous injections of living *Staphylococcus aureus*. His earliest positive result was in a rabbit after 21 days.

Schepilewsky (19) produced extensive subcutaneous necrosis and suppuration by the injection of various ferments. Out of a considerable number of experiments he reports positive results in two rabbits injected with lab-ferment, in one injected with pancreatin, and in one with papayotin. The author makes the criticism of the positive results of Lubarsch and Nowak with turpentine, and of those of Krawkow, who obtained a positive result in one rabbit with *Bacillus pyocyaneus* toxin, and of Davidsohn, who obtained a positive result in one mouse injected with dead bacteria, that bacterial contamination of the suppurating lesions produced was not excluded. Davidsohn (14, 16) on the other hand, believing that amyloid cannot be produced without the action of living bacteria or their products, makes a similar criticism of Schepilewsky's results with ferments, and apparently with equal justice. The latter admits that the abscesses in one of the four positive cases were not sterile. The other three he believes were sterile, but each of these animals died of an epidemic disease of a nature not stated, and one of the three had a purulent bronchitis.

Dantschakow (20) studied the salivary glands of rabbits injected subcutaneously with living *Staphylococcus aureus*. She believes that amyloid first appears here. She found it in some cases in the 2nd week, in others not until the 6th or 7th.

Tarchetti (21) reports entirely negative results. He injected a dog subcutaneously with *Staphylococcus aureus*. When the animal had become immune and the injections no longer produced abscesses, he used turpentine. The duration of the experiment was 4 months. Three rabbits injected subcutaneously with turpentine and eight with living *Staphylococcus aureus* were negative. He concludes that repeated injections of turpentine or broth cultures of *Staphylococcus aureus* produced no amyloid in the spleen of dogs, rabbits, or guinea pigs.

Ravenna (22) concludes from many experiments that the question of experimental production of amyloidosis still cannot be considered as solved, as a means is lacking which will produce it with certainty.

Of special interest in connection with the experiments here reported are the

observations of amyloid in the liver of horses repeatedly injected with bacteria or their toxins for the production of immune serum.

Zenoni (23) first reported the presence of amyloid degeneration in diphtheria antitoxin horses, especially in the liver. The lesion produced a very friable organ and death was apt to occur from spontaneous rupture and hemorrhage.

Pease and Pearce (24) report liver necrosis and venous thrombosis in twelve horses which had been repeatedly injected subcutaneously, seven with diphtheria toxin, two with tetanus toxin, one with dysentery bacilli, and two with streptococci. A horse is also added to the series which had been injected with diphtheria toxin and killed on account of its crippled condition. Its organs were normal. Two of these horses, both injected with diphtheria toxin, showed amyloid in the liver and spleen, especially in the latter. It was not present in the kidneys, heart, or lymph nodes. They note that these two animals had developed an unusual number of abscesses at the sites of injection, apparently attributing the amyloid formation to this fact.

Lewis (25) reports that amyloid degeneration of the liver and less frequently of the spleen is produced in a majority of horses by the routine injection of diphtheria toxin and repeated bleeding, extending over a period of 3 years. The horses are apt to die from rupture of the liver and intraperitoneal hemorrhage. He rules out abscesses as an essential condition and also the repeated bleeding, and concludes that the crude toxin is the factor of prime importance in the production of amyloid.

Schoukewitch (26) reports that repeated injections of large doses of *Bacillus pestis* produce amyloid degeneration of different organs in horses and that pus formation is not essential.

Markus (27) observed amyloid in the liver of horses injected with dysentery bacilli.

#### EXPERIMENTAL PART.

In the following experiments rabbits were injected in the ear vein with beef extract broth cultures of colon bacillus, transferred from stock cultures on beef extract agar. The rabbits were injected, generally every 2 to 4 days, with 1 cc. of a 24 to 48 hour culture, though older cultures were occasionally used. Colon S 232 and Colon S 244 were strains of *Bacillus coli communior* obtained in pure culture from surgically removed pyonephrotic kidneys. Colon B was also *Bacillus coli communior* isolated from a normal stool.

Frozen sections of tissues fixed on Orth's fluid were stained with hematoxylin and Sudan III and also by Van Gieson's method. Sections of paraffin-imbedded tissues were also stained by Van Gieson's method. Tissues were stained for amyloid with iodine, iodine and sulphuric acid, methyl violet, and gentian violet. As has

been previously mentioned, the finding of amyloid in these rabbits was unexpected. The hyaline bodies in the kidney glomeruli and other glomerular and parenchymatous changes had been noted, but that the bodies in the glomeruli were of amyloid nature was not recognized until a majority of the rabbits had come to autopsy and pieces of their organs had been fixed in Orth's fluid only. Consequently it was generally possible to try the various reactions only under the relatively unfavorable conditions of previous fixation in Orth's fluid. In one rabbit, No. 15, the reactions were tried on fresh and alcohol-fixed tissues; in Rabbits 1, 3, and 7, on alcohol-fixed tissues; in all others, on tissues fixed in Orth's fluid only. The reactions were tried under these conditions on the spleen, liver, and kidneys of all rabbits. Pieces of other organs, however, which are of interest in connection with amyloidosis had not been preserved in most cases, and it is therefore impossible to report the presence or absence of amyloid degeneration in other situations except in a few instances. Table I shows the strain of bacteria used, the duration of the experiment in days, the number of injections, and the presence or absence of amyloid in the spleen, liver, and kidneys. Positive findings in other organs will be mentioned later.

TABLE I.

Rabbit No.	Bacteria.	Duration of experiment.	No. of injections.	Amyloid.
		<i>days</i>		
1	Colon S 232	21	7	None.
2	" S 232	26	9	"
3	" S 232	30	11	"
4	" S 232	30	10	"
5	" S 244	59	8	"
6	" S 232	61	15	"
7	" S 232	63	19	"
8	" S 232	88	23	Spleen, liver, kidney.
9	" S 232	98	25	"
10	" S 232	102	25	"
11	" S 232	113	29	" liver, kidney.
12	" B	115	27	" kidney.
13	" S 232	116	36	" "
14	" S 232	142	30	" "
15	" S 232	145	47	" liver, <sup>7</sup> / <sub>8</sub> kidney.

Complete autopsies were performed on all the animals, which, however, will not be reported in detail, as the gross pathological findings were few. Suppurative lesions were lacking with the following exceptions: Rabbit 3 had an abscess about 1.5 cm. in diameter in the upper anterior mediastinum, and Rabbit 14 had a few small nodular areas of consolidation in the lungs, each about 3 mm. in diameter with central softening. All the rabbits lost much in weight, emaciation being extreme in the older cases. The livers were of about normal size and no change in consistency was noted except in No. 15, in which the organ was definitely firmer than normal but more friable. All the spleens were somewhat enlarged; in No. 14 the organ was about four times normal size, in No. 9 about three times, in Nos. 1 and 11 about twice; in the others the enlargement was slight. Beyond the enlargement there was no marked change in appearance or consistency. The gross appearance of the kidneys was not striking. In all the animals they were definitely opaque, in many very pale, and in some apparently swollen. Small, slightly depressed, purple or gray surface scars were present in many instances, in some being moderate in number, in most very few. None were present in Nos. 1, 2, 3, 4, 5, and 7. They had the same appearance as the spontaneous scars in rabbits, and it is difficult to decide whether they are to be ascribed to the treatment or to a preexisting spontaneous nephritis. The fact that they were absent in the experiments of shorter duration would favor the former view. The number and severity of the scars when present, however, definitely bore no relationship to the length of time the rabbit had been injected, the scars in Nos. 6, 9, and 10 being more numerous than in the older rabbits, in which they were very few and slight.

Microscopical examination of the spleens of the earlier rabbits, Nos. 1 to 7 inclusive, showed a large amount of hematogenous pigment collected in large phagocytic cells in the sinuses of the pulp. In some there was a slight diffuse fibrosis. Otherwise the spleens were normal. In Rabbits 8 to 15 the hematogenous pigment was also present and in all there was a fibrosis. All showed about the Malpighian bodies a homogeneous hyaline substance which in the more marked cases, Nos. 8, 11, 12, and 13, encroached on these structures (Fig. 4) and almost entirely replaced them, only a small

collection of lymphoid cells remaining grouped about the central artery. A moderate amount of nuclear detritus was present in this material, and in No. 8 numerous fairly large hemorrhages were present in the midst of the hyaline material, which had largely replaced the Malpighian bodies. Similar hyaline material was also seen in some animals in the reticulum of the pulp. In Rabbits 14 and 15 the fibrosis and hyaline formation, though more marked about the Malpighian bodies, was also present through the reticulum of the pulp (Fig. 5). Stained with methyl violet or gentian violet, this hyaline material, both about the Malpighian bodies and in the pulp, gave a typical red amyloid reaction in all cases (Fig. 1). It appeared in the form of small irregular masses which usually were quite homogeneous, but sometimes suggested a finely granular structure. When present in the reticulum of the pulp it had a definite fibrillar appearance. With iodine no reaction was obtained even in the fresh and alcohol-fixed tissues of No. 15. With iodine followed by sulphuric acid the larger collections about the Malpighian bodies gave a pale but definite greenish color. These reactions will be considered later. Large phagocytic cells were quite numerous in the spleen of these rabbits, and in No. 15 fairly numerous giant cells were present, having many nuclei and often including masses of the amyloid (Fig. 1). These cells have been previously noted by Krawkow (7).

All the livers showed a definite but usually moderate increase of periportal connective tissue and also an infiltration with round cells in these areas. It is difficult to decide whether this is a spontaneous cirrhosis or is due to the treatment. There were also present in all the animals a varying number of small focal necroses situated indiscriminately in the lobules. In the earlier ones these were infiltrated with round cells; in the later ones they were often replaced by connective tissue. There was considerable granular degeneration of the liver cells in these rabbits and also pyknosis of nuclei. In No. 13 numerous large central necroses were present with much fibrin and some hyaline degeneration but little cellular infiltration. The livers of Rabbits 8, 11, and 15 showed between the walls of the capillaries and the trabeculae of liver cells a layer of hyaline material with atrophy and granular degeneration of the intervening liver cells. This was present in all lobules, but in No. 8 was seen only in the peripheral and middle



zones of the lobules, while in the other two it was fairly uniform throughout. With iodine and iodine followed by sulphuric acid, no reaction was obtained. With methyl violet and gentian violet this material gave a typical amyloid reaction. With these stains the substance appeared usually in a definite granular form (Fig. 2), but sometimes a fine fibrillar structure was apparent. The necroses with hyaline degeneration described in No. 13 gave no reaction. No amyloid was found in the walls of the larger vessels.

The kidneys of these rabbits deserve special attention, since they showed not only amyloid deposits but also other lesions definitely due to the bacterial injections. Degenerative changes were present in the tubular epithelium in all cases. Frozen sections stained with Sudan III showed a moderate fatty degeneration affecting principally the convoluted tubules. The fatty degeneration was, however, more marked in the earlier than the later cases. There was a definite granular degeneration, also most marked in the convoluted tubules, and some necrosis of the epithelium with pyknosis of nuclei, lack of nuclear staining, and desquamation. The large granules seen in the epithelium of the proximal convoluted tubules in chronic parenchymatous nephritis in man were not observed. Casts were present in all kidneys except those of Nos. 1 and 2, and in Nos. 13, 14, and 15 they were numerous. No glomerular changes, other than hyperemia, were noted in Rabbits 1 to 7, while all the others showed evidences of a subacute and chronic glomerulitis. The lesions consisted of fibrous and hyaline thickening of the vascular loops and occasionally of the glomerular capsules, protoplasmic, cellular, and fibrous adhesions between the tufts and capsules, areas of necrosis in the tufts, and hyaline bodies containing a few pyknotic nuclei and some nuclear débris. These lesions varied in number and degree in the different rabbits. Adhesions were present in Nos. 8 to 15 inclusive, being, however, few in Nos. 9 and 12 but numerous in the others, especially Nos. 13 and 14 (Fig. 6). Almost every glomerulus in No. 13 showed adhesions. Some of these adhesions were fine protoplasmic unions between tuft and capsule (Fig. 7), others large and cellular or fibrous (Figs. 7 and 8). Fibrous and hyaline thickening of the vascular loops was present in Nos. 8, 11, 12, 13, 14, and 15, with necroses and large amyloid bodies in some instances (Figs. 7, 8, and 9). The fre-

quent occurrence of localized interstitial scars in the kidneys of rabbits unfortunately renders the interpretation of interstitial lesions in these animals difficult. As has been already mentioned, most of the rabbits which withstood the injections longest showed a few small scars in the gross. There were usually only two or three such scars on a kidney. These scars have been disregarded as perhaps due to a preexisting spontaneous nephritis. In most of the kidneys, however, there were present small collections of round cells, and in Nos. 13, 14, and 15 there was a slight, rather diffuse, cellular thickening of the interstitial tissue. It is believed that these lesions were produced experimentally.

Six of the kidneys, as shown in the table, gave a typical amyloid reaction with methyl violet. The amyloid was present in the glomeruli as small and large nodular collections (Fig. 3) and also in the medulla where in some instances it was situated only about the small capillaries in small localized areas; in others it involved the entire interstitial tissue, in similar small nodular areas, here surrounding both vessels and tubules. No amyloid was noted in the larger vessels. Here as in other organs it was difficult to convince one's self definitely of the morphology of the amyloid. In certain instances the large deposits in the glomeruli seemed to have a definite fibrillar structure with a frayed out appearance at the edges, especially noticeable in the alcohol-fixed tissue of No. 15. In some of the other cases these bodies appeared quite homogeneous (Fig. 3). With iodine no reaction was obtained even with frozen sections of the fresh tissue of No. 15. With iodine and sulphuric acid the larger bodies in the glomeruli, like the deposits in the spleen, gave a pale but definite greenish reaction.

By reference to the table one notes that amyloid was present in the spleen of all rabbits, eight in number, which were injected for a period of 88 days or longer. These eight rabbits showed glomerular and other lesions in the kidneys, and in six amyloid was present. The livers of three contained amyloid. Organs other than the spleen, liver, and kidneys were examined for amyloid in only a few instances. The stomach, small intestine, large intestine, heart, adrenal, and submaxillary gland in No. 3 gave no reaction. The small intestine, large intestine, heart, and submaxillary gland in No. 7 gave no

reaction. The stomach and small intestine of No. 15 showed typical deposits in small scattered areas in the mucosa only; small irregular homogeneous bodies in the bone marrow gave a doubtful reaction with methyl violet; the heart and adrenals showed none.

#### DISCUSSION.

It seems justifiable to regard the deposits described in these animals as amyloid in spite of the absence of the iodine reaction. It was possible to try the reaction on fresh or alcohol-fixed tissues in only one, No. 15, of the eight rabbits which showed deposits. There is a possibility that the reaction might have been obtained in some of the other animals under these conditions. It is the consensus of opinion, however, of those who have studied the question carefully, that the reaction with aniline colors is the more typical and that the iodine and iodine-sulphuric reactions are sometimes lacking. Davidsohn (11) and others believe that these reactions represent different stages in the development of amyloid, the reaction with aniline colors appearing earliest and being present throughout, the iodine reaction appearing next, and that with iodine and sulphuric acid representing the latest stage. Krawkow (10) and Maximow (18) report results similar to the above with the amyloid experimentally produced by them in animals. Krawkow (10) found the reaction with methyl violet constant, but obtained the iodine reaction in only a few instances, and then only on fresh or alcohol-fixed tissues, and in the latter it disappeared after 24 hours' fixation. Similar absence of the iodine reaction in amyloid deposits in man has been reported by Hanse-mann (28), Krawkow (10), and others. Krawkow (10) believes that these reactions do not represent a difference in chemical composition of the substance, but are due to a difference in physical conformation of the older deposits. The presence of the iodine-sulphuric reaction in the absence of the simple iodine reaction appears from the record of previous experiments to be exceptional. The possibility suggested itself that the green color obtained in these cases might be due to the presence of cholesterol in these degenerated areas. The large deposits in the kidneys and spleen, however, give no suggestion of a fat reaction with Sudan III, as one would expect were

the cholesterol present in any considerable amount in lipid form; and the fact that the reaction was also obtained on specimens imbedded in paraffin would seem to rule out cholesterol as the responsible factor.

Scrapings of the fresh liver, spleen, and kidneys of Rabbits 3, 6, 8, 11, 13, and 14 were examined with the polarizing microscope for anisotropic fat. This examination is of interest on account of the reports of the presence of doubly refracting lipoids in human nephritis, particularly in the chronic parenchymatous type which these kidneys so closely resemble. The results were negative except in the liver of No. 6 and the kidneys of No. 8, in both of which a moderate number of small anisotropic droplets were present.

One notes that in the spleen there is an association of amyloid formation with evidences of blood destruction, cell necrosis, and fibrosis. Also in the glomeruli of the kidneys one sees necrosis and fibrosis associated with the deposition of amyloid. In the liver this association is not so evident. It is not justifiable to draw definite conclusions as to a possible relationship between amyloid formation and any one of these other phenomena. While the cell destruction may in part be the result of the deposition of amyloid, or the two may be mutually independent results of the toxic action, we believe that the possibility of a genetic relationship between cell necrosis and the formation of amyloid should not be too readily dismissed. Mallory's views are worthy of attention in this regard. He states (29) that amyloid in man when first deposited is finely fibrillar but soon becomes homogeneous and that it is not a product of degeneration of cell or fibril, nor is it something filtered out of the blood-like serum, but it is an abnormal product of the fibroblast. His argument that it is more reasonable to regard amyloid as the product of the fibroblast, which normally produces fibrils, than of the endothelial cell which does not, is forcible. He also calls attention to the fact that chondroitin sulphuric acid is a constituent of some of the normal products of these cells; namely, elastic fibers and chondromucin. As between its formation by endothelial cells and fibroblasts, these facts are certainly in favor of the latter. As previously noted, its fibrillar appearance in the spleen and kidneys of these rabbits is sometimes quite evident. This fibrillar appearance, however, does not seem to the writer good evidence that it is produced

as such by either fibroblasts or endothelial cells. The variation in the morphological appearance of the amyloid in different situations in these rabbits has already been noted. One is also impressed in reviewing the literature with the variations in appearance as described by different authors, some believing it to be fibrillar or homogeneous, others granular, and others crystalline. Of the various theories advanced as to the nature of this substance, the idea that it is a homogeneous, semisolid, infiltrating substance would seem to explain these variations in morphological appearance better than any other, variations being produced, as they are particularly apt to be in colloidal materials, by postmortem changes or the processes of fixation, and being less easily produced in older deposits, perhaps because of greater inspissation. If such a substance were produced or deposited in narrow tissue spaces, it might well appear in fixed specimens as thin sheets or fibrils. With atrophy of intervening collagen fibrils, which Mallory describes as occurring following the appearance of the amyloid fibrils, the infiltrating material might well fuse to form the homogeneous masses of the later stages.

Living bacteria only were tried in the experiments here reported. That the injection of dead colon bacilli alone might result in amyloid deposits is suggested by the results of Davidsohn (16) on a mouse injected with dead bacteria, and that toxins or decomposition products of the culture media might produce it is suggested by the results of Krawkow (7) and Nowak (17), each on a rabbit injected with filtrate of a culture of *Bacillus pyocyaneus* and by those on horses immunized with diphtheria toxin. These are questions not answered by these experiments. That it could be due to the normal constituents of beef extract broth would seem improbable. That this is not the case is evidenced by Rabbit 6 which had received twenty-five intravenous injections of sterile broth in a period of 101 days before the bacterial injections were begun. The period of injection of sterile broth was thus not as long as that of some of the rabbits which showed amyloid, but longer than in two of the rabbits, and about the same as that of a third which showed definite amyloidosis.

The question as to whether the ability to produce amyloidosis is a common property of all pathogenic bacteria or is confined to certain species or classes, is of considerable practical interest and also

one which has not been answered. From a study of previous work it seems evident, in spite of conflicting reports, that subcutaneous suppuration produced by living *Staphylococcus aureus* in hens and rabbits may, especially in the former, ultimately result in the formation of amyloid deposits. The positive results reported with other organisms are so few and the variety of animals on which these results were obtained is so great, that it is difficult to draw conclusions in this regard. The criticism made of the experiments in which subcutaneous suppuration has been produced by turpentine, ferments, and bacterial toxins,—that bacterial contamination was not excluded,—seems justified. The difficulty of keeping such subcutaneous lesions uncontaminated, especially with staphylococcus which we know will produce amyloid, over periods of weeks or months is obvious. Attention has already been called, in reviewing the literature, to the contradictory reports as regards the possibility of producing amyloidosis by subcutaneous suppuration and also to the inconsistency, as far as time is concerned, in the experiments of the various investigators who have reported positive results. In contrast with previous work the constant results here reported suggest the importance of bacteriemia in the production of amyloid and afford a possible explanation of the varying results in previous experiments, that in some cases bacteriemia resulted while in others the infection remained localized. Certain results reported, particularly those on horses, indicate that a toxemia is sufficient, without actual bacteriemia.

The author has previously stated that the experiments here reported with colon bacilli are a part of a larger series in which various bacteria were used. The only rabbit injected with other varieties of bacteria which lived 88 days or longer (that is, longer than the shortest period in which amyloid was produced with colon bacilli) was one injected with *Bacillus typhosus*. This animal received forty injections of 1 cc. each of broth culture in a period of 125 days. Another rabbit received twenty-three injections of 1 to 2 cc. each in a period of 84 days. Neither of these rabbits showed amyloid deposits. One would not be justified from these two instances in drawing definite conclusions, yet the results would seem to indicate that at least there is a marked difference in the facility with which amyloid is produced by different organisms. The principal other bacteria used were

various strains of streptococci. Amyloid was not produced, but inasmuch as the duration of the longest experiments was 87 days (ten injections) and 78 days (twenty-two injections) comparative conclusions in these instances also are not warranted. If experimental investigation should prove that amyloid is produced more easily by some varieties of organisms or their products than by others, a careful investigation of the numbers and varieties of bacteria present in pulmonary cavities in chronic tuberculosis would also be of interest as possibly affording an explanation of the recognized fact that amyloidosis is extreme in some cases of chronic tuberculosis while entirely lacking in others of equal duration and extent of lesions. The explanation which has been offered of individual and racial susceptibility seems inadequate to explain the marked differences seen in this regard. At least the explanation is one to which we should not resort until the above possibility has been excluded.

#### SUMMARY.

The repeated intravenous injection of rabbits with living *Bacillus coli communior* over long periods has resulted in the formation of amyloid deposits in the spleen, liver, and kidneys. Suppurative lesions were not present in most cases and therefore not a factor in its production. The results have been constant in that amyloid was found in all rabbits, eight in number, which were injected over a period of 88 days or more. Eight rabbits showed amyloid in the spleen, six of these in the kidneys also, and three in the liver.

The kidneys of these eight rabbits also showed as a result of the injections a subacute and chronic glomerulitis, parenchymatous degeneration, some interstitial infiltration with round cells, and a slight cellular proliferation of connective tissue, thus resembling the chronic parenchymatous nephritis of man which is so commonly associated with amyloid disease.

In conclusion I wish to express my appreciation of the advice and assistance of Dr. Ophüls throughout this work.

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## EXPLANATION OF PLATES.

## PLATE 110.

FIG. 1. Rabbit 15. Spleen. A portion of a Malpighian body is shown which was surrounded and partially replaced by amyloid. A giant cell is seen, at the upper end of the picture, with amyloid inclusions. Camera lucida water color drawing by Dr. F. E. Blaisdell. Zeiss obj. E., oc. 1. Stained with methyl violet.

FIG. 2. Rabbit 15. Liver. The picture shows a considerable deposit of amyloid between the capillary walls and liver cells with granular degeneration and atrophy of the latter. The amyloid has a finely granular appearance. Camera lucida water color drawing by Dr. F. E. Blaisdell. Zeiss obj. E., oc. 1. Stained with gentian violet.

## PLATE 111.

FIG. 3. Rabbit 14. Kidney. The upper glomerulus was of homogeneous appearance with loss of normal structural detail and contained many pyknotic nuclei and a large homogeneous appearing amyloid body. The lower glomerulus showed a general thickening of the vascular loops with two small amyloid nodules. There were adhesions in both cases between tuft and capsule. Camera lucida water color drawing by Dr. F. E. Blaisdell. Zeiss obj. E., oc. 1. Stained with methyl violet.

## PLATE 112.

FIG. 4. Rabbit 12. Spleen. A Malpighian body is shown surrounded and encroached upon by amyloid formation; also thickening of and deposition of amyloid in the reticulum of the pulp. Microphotograph. Bausch and Lomb obj.  $\frac{3}{4}$ , oc. 1.

FIG. 5. Rabbit 14. Spleen. The photograph shows a fibrosis affecting the Malpighian body and also the reticulum of the pulp. There is some amyloid about the Malpighian body, but it is relatively more pronounced in this case in the reticulum of the pulp. Microphotograph. Bausch and Lomb obj.  $\frac{3}{4}$ , oc. 1.

## PLATE 113.

FIG. 6. Rabbit 13. Kidney. The photograph shows eight glomeruli in each of which there are adhesions between tuft and capsule. Two glomeruli, the one at the top of the picture in the middle and also that at the bottom, show large amyloid bodies. Microphotograph. Bausch and Lomb obj.  $\frac{3}{4}$ , oc. 1.

## PLATE 114.

FIG. 7. Rabbit 13. Kidney. A higher magnification of the glomerulus seen at the top of Fig. 6. Note the fine protoplasmic and large cellular adhesions, the hyaline body, and the swelling and slight proliferation of the lining cells of the capsular space. In an adjacent section of this same glomerulus the hyaline body gave a typical amyloid reaction with methyl violet. Microphotograph. Bausch and Lomb obj.  $\frac{3}{4}$ , oc. 1.

## PLATE 115.

FIG. 8. Rabbit 13. Kidney. A glomerulus from the same rabbit but not in the field of Fig. 6. Note the extensive adhesions and the large hyaline body containing some nuclear débris. In an adjacent section the hyaline body in this glomerulus gave a typical amyloid reaction with methyl violet. Microphotograph. Bausch and Lomb obj.  $\frac{3}{4}$ , oc. 1.

FIG. 9. Rabbit 15. Kidney. Note the large body in the glomerulus with a few pyknotic nuclei and some nuclear débris. In an adjacent section of this same glomerulus the entire body gave a typical amyloid reaction, the material appearing in a distinctly fibrillar and finely granular form. Microphotograph. Bausch and Lomb obj.  $\frac{3}{4}$ , oc. 1.

FIG. 1.

FIG. 2.

(Bailey: Amyloid Disease and Chronic Nephritis.)



FIG. 3.

(Bailey: Amyloid Disease and Chronic Nephritis.)



FIG. 4.

FIG. 5.

(Bailey: Amyloid Disease and Chronic Nephritis.)





FIG. 6.

(Bailey: Amyloid Disease and Chronic Nephritis.)



FIG. 7.

(Bailey: Amyloid Disease and Chronic Nephritis.)



FIG. 8.

FIG. 9.

(Bailey: Amyloid Disease and Chronic Nephritis.)



## BACTERIOLOGICAL AND EXPERIMENTAL STUDIES ON GASTRIC ULCER.

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Many investigators have attempted to produce chronic gastric ulcers experimentally. These experiments have been performed on various species of animals, and a great variety of methods has been used in causing the original injury to, or defect in the gastric mucosa. All these attempts have failed, and the continuity of the gastric mucosa was always restored by prompt healing unless the procedure caused a complete stoppage of the blood supply to a part of the stomach. This was followed in some instances by infarction and by perforation and its sequelæ.

Recently Rosenow<sup>1</sup> has published some interesting investigations on the production of acute and chronic gastric ulcers in rabbits and dogs. His full report has not yet appeared. He isolated anhemolytic streptococci, so called *Streptococcus viridans* or *mitis*, from 96 per cent of a series of gastric ulcers removed from human subjects at operation. Recently isolated cultures were injected intravenously into animals, and in 60 per cent gastric ulcers were found. In a few animals which were allowed to live for a considerable time after their inoculation, in some instances several months, chronic ulcers were found at autopsy. From these results Rosenow has reached certain conclusions which may be briefly summarized as follows:

1. Anhemolytic streptococci can be recovered by a special technique from practically all gastric ulcers removed at operation.
2. The streptococci from this source possess a specific affinity for the stomach which enables them to localize in this organ, when recently isolated cultures are injected intravenously into animals.
3. About 60 per cent of the animals inoculated in this manner develop gastric

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<sup>1</sup> Rosenow, E. C., and Sanford, A. H., *Jour. Infect. Dis.*, 1915, xvii, 219; *Jour. Ind. State Med. Assn.*, 1915, viii, 458; *Jour. Am. Med. Assn.*, 1915, lxxv, 1687.

ulcers. Other lesions also occur following these inoculations, but with less frequency.

4. Streptococci can be recovered from these experimental ulcers and can be demonstrated histologically. They reach this location by the blood stream and are deposited in the capillaries of the gastric mucosa.

5. These streptococci are identical with those inoculated.

6. Anhemolytic streptococci are, therefore, the cause of gastric ulcers in man, and these organisms reach the stomach by a hematogenous route.

The following experiments were undertaken following Dr. Rosenow's presentation of his investigations in New York in April, 1915. The technique which we employed in isolating and injecting the streptococci is identical with his. We are indebted to Dr. Rosenow for having furnished us with a description of his technique before the publication of his work.

#### *Material.*

Eight chronic gastric ulcers removed at operation from human subjects were examined bacteriologically and histologically. We have also studied one ulcer occurring at the ostium of a gastrojejunostomy. The results obtained from these examinations and the animal experiments performed with the organisms recovered from these ulcers form the basis of the following report. Although the number of specimens studied has not been large, the consistency of the results obtained by all three methods of investigation seems to us to justify the present communication.

The eight gastric ulcers studied were all of the chronic indurated type. All the cases from which they were removed had typical gastric symptoms which varied in duration from 2 months to 8 years. Two of the ulcers were located at the pylorus and six on the lesser curvature.

On examination of the stomach at operation the ulcer-bearing area was found thickened and indurated and either a defect in the mucosa or a crateriform ulcer was felt.

Brief descriptions of the gross appearance of the specimens examined follow.

A. Small piece of ulcer removed. Ulcer felt crateriform to operator.

C. Funnel-shaped ulcer of the greater curvature, near pylorus, 1.5 cm. in width, with firm fibrotic margins.



D. Ulcer of the lesser curvature, 1.5 cm. in width, extending through the entire gastric wall into the gastrohepatic omentum. Edges overhanging, firm, and fibrotic.

E. The same appearance as D.

G. Ramifying, irregular ulcer involving the lesser curvature, the entire pyloric antrum, and the first cm. of the duodenum. It has a punched out appearance with sharp edges, not overhanging; the base extends into the muscularis.

J. At the middle of the lesser curvature of the pyloric antrum there is an ulcer 2 cm. in width, perforating entirely through the stomach wall into the gastrohepatic omentum, with sharp, firm edges that do not overhang.

L. Round, punched out ulcer, 1 cm. in width, with firm edges which slightly overhang. The ulcer extends almost through the entire wall of the stomach.

M. The specimen consists of a segment of the middle 7 cm. of the stomach. The middle of the posterior wall is occupied by a perforated ulcer, 3 cm. in diameter, with sharp edges.

*Gastrojejunal Ulcer.*—This specimen was removed from a patient on whom 3 years previously a posterior gastrojejunostomy by the Murphy button method had been performed, together with a pyloric exclusion by the string method for a perforated duodenal ulcer situated just beyond the pylorus.

*Operation and Specimen.*—At the site of the former duodenal ulcer there is an indurated area. The string is still in place and the pylorus almost completely closed, barely admitting the tip of the little finger. The gastrojejunostomy opening is considerably stenosed and admits only the tip of the little finger. The edge of the ostium appears soft and normal over about two-thirds of its circumference. The remaining one-third feels indurated and the serous surface over this is bright red in color and shows a few very recent, fibrinous adhesions between the jejunum and the transverse mesocolon. The anastomosis between the jejunum and the stomach was separated with difficulty. Beneath the reddened portion of the ostium was found what appeared to be a definite and somewhat indurated ulcer of the stomach, 1.5 cm. in size. This portion was resected and a new gastrojejunostomy was performed at the same site, by the suture method.

### *Methods.*

Immediately after the operation pieces of ulcer were removed for culture and the remainder of the material was fixed in 70 per cent alcohol. Some of the pieces were incubated in deep tubes of glucose-serum-bouillon for 24 and 48 hours and then fixed in alcohol. Sections were prepared by both the paraffin and celloidin methods and stained with hematoxylin and eosin, Giemsa and Unna-Pappenheim stains, and by the Gram-Weigert method.

Formalin fixation was not used as this interferes with the demonstration of bacteria. The aniline oil, gentian violet used in the Gram-

Weigert method was always filtered through Berkefeld filters to remove any bacteria present which might be deposited on the section and lead to confusion.

### *Microscopical Studies.*

Microscopical examination reveals a great similarity in the pathological processes in the specimens. All the ulcers show certain prominent characteristics. The edge of the ulcer is sharp, as though cut off, even when it is overhanging. The mucosa at the edge shows only a superficial degeneration and a moderate grade of chronic inflammation consisting of congestion, edema, and diffuse infiltration with mononuclear leukocytes, plasma cells, and a few polymorphonuclear leukocytes. The glandular structures at the edge of the ulcer show slight degenerative changes of their cells. Some of the glandular acini or crypts also have the appearance of being cut off and do not reach the surface of the mucosa but terminate at the edge of the ulcer. Whenever found, the cells of the acini are contained within their basement membrane and show no tendency to hyperplasia or malignant transformation. The lining, *i. e.*, the surface of the ulcer, consists of a narrow layer from one-eighth to one-third the diameter of a low power field of the microscope in thickness and resembles very much the degenerating tissue which is found situated in tuberculous lesions between the areas of caseation and the surrounding tuberculous granulation tissue. The layer of degenerating tissue fuses with or is transformed into dense fibrous tissue, although the line of demarcation is clearly seen. The fibrous tissue extends for a considerable distance, in places as much as 1 cm., into the submucosa and muscularis. It has the typical characteristics of scar tissue and seems to form a wall beneath the ulcer. Some of the ulcers have completely penetrated the stomach wall, but the edges are always covered by the above structures and the bare muscularis is never exposed. The connective tissue septa which lie between the muscle bundles and are accompanied by blood vessels, are increased in size by fibrous tissue and dense infiltration with mononuclear leukocytes. Similar areas of fibrosis and infiltration are found beneath and in the serosa.

Occasionally, but only rarely, prolongations of the degenerat-

ing surface layer, several mm. in size, are found in the dense fibrous tissue layer, but these are always completely limited by the latter.

With the special stains for bacteria, especially with the Gram-Weigert stain, organisms are found only in or upon the lining of the degenerating tissue but never in the depths of this except in the prolongations described above. The organisms usually occur in small clumps or as sparsely scattered individuals, and are not found in the dense fibrous layer, the stomach wall, the connective tissue septa, or the areas of leukocytic infiltration of the serosa. The chief organisms found are minute cocci (streptococci), peculiar forms of yeast, and various types of bacilli. The same forms are found in great numbers throughout the tissue which was incubated in bouillon before it was fixed in alcohol, but are most numerous on and near the surface of the ulcer. The types of organisms observed in the preparations from the material fixed immediately and the organisms recovered in cultures are given in parallel columns in Table I.

In all, 109 tubes were inoculated either with emulsions or pieces of ulcer. Streptococci were present in 32 tubes, occurring in pure culture in 11. 13 tubes remained sterile, and the other 64 tubes contained various types of organisms.

The streptococci were anhemolytic and had the following morphological and cultural characteristics.

*Morphology.*—Stained preparations. Small, Gram-positive cocci, forming short and long chains. No capsule. Hanging drop of growth in deep tubes of glucose-serum-bouillon shows long convoluted chains.

*Bouillon.*—Slight diffuse cloudiness; many granular clumps adherent to the sides of the tube and also deposited at the bottom as sediment.

*Litmus Milk.*—Acidified but not coagulated.

*Gelatin.*—Not fluidified.

*Inulin-Serum-Peptide-Water-Medium.*—Not fermented.

*Bile.*—No solution of organisms.

*2 Per Cent Glucose-Serum-Agar.*—Dry, granular, fairly profuse growth. All strains cause precipitation, most of them marked precipitation.<sup>2</sup>

*Plain Agar.*—Sparse, dry growth, mostly as minute, discrete, pearly white colonies.

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<sup>2</sup> Libman, E., *Jour. Med. Research*, 1901, i, 84.

TABLE I.

Ulcer.	Types of organisms found in sections of ulcers.	Organisms recovered in cultures from ulcers.
A	Few groups of round bodies, suggestive of cocci, upon surface of ulcer.	Streptococci and several unidentified bacilli. No pure cultures. 6 tubes inoculated; 2 contained streptococci.
C	Small groups of cocci, few bacilli, some of them diphtheroids, found in and on degenerating lining near edge of ulcer.	Streptococci, <i>Bacillus subtilis</i> , <i>Micrococcus tetragenus</i> , large Gram-positive bacilli, diphtheroid bacilli. 8 tubes inoculated. Streptococci pure in 1 and together with bacilli in another.
D	Few clumps of 15 to 20 small cocci on surface of ulcer.	Streptococci, yeast, <i>Staphylococcus albus</i> , Gram-positive and Gram-negative motile bacilli, Gram-positive non-motile bacilli. 14 tubes inoculated, 2 of which contained pieces of ulcer. In one of the latter streptococci were found in pure culture; in 1 other tube they occurred together with Gram-positive bacilli.
E	Large numbers of organisms singly and in clumps found on degenerating surface of lining of ulcer. These are mostly small cocci in long chains; many large bacilli and a few slender bacilli.	Streptococci were seen in cultures but could not be isolated; slender Gram-positive bacilli, staphylococci, spore-bearing Gram-positive bacilli, and a pleomorphic Gram-positive bacillus were isolated. 13 tubes inoculated, 2 of which contained pieces of ulcer. In the latter cocci in long chains were seen; 4 tubes were sterile.
G	Few organisms in and upon degenerating lining of ulcer. These are mainly large and small bacilli and a few cocci.	Streptococci, Gram-positive bacilli, motile Gram-negative bacilli, <i>Staphylococcus albus</i> , <i>Bacillus subtilis</i> , Gram-negative spore-bearing bacilli. 14 tubes inoculated, 2 of which contained pieces of ulcer. In 1 of the latter streptococci were found in almost pure culture and in the other there were a few chains of streptococci and Gram-negative motile bacilli. Of the remaining 12 tubes, 1 contained streptococci in pure culture, and 4 contained streptococci together with 1 or more other organisms.

TABLE I—*Concluded.*

Ulcer.	Types of organisms found in sections of ulcers.	Organisms recovered in cultures from ulcers.
J	Few bacilli on surface of ulcer. In a prolongation of the necrotic lining of the ulcer into the depth there are yeasts, scattered and in large clumps; small cocci, some of which are in chains; large cocci.	Streptococci, yeast, staphylococci, <i>Bacillus subtilis</i> , spore-bearing Gram-negative bacilli. 16 tubes inoculated, 2 with pieces of ulcer, which contained streptococci together with Gram-positive bacilli. In 3 of the remaining tubes streptococci were found in pure culture, in 4 together with other organisms, and 1 tube was sterile.
L	In and upon the degenerating lining are found masses of yeast but no bacteria.	Yeast, <i>Staphylococcus albus</i> , small Gram-positive bacillus. 12 tubes inoculated. None contained streptococci, but yeast and <i>Staphylococcus albus</i> were recovered from all.
M	In and upon the degenerating lining are many cocci, some in chains. There are as many bacilli present as cocci; some of the bacilli are large and in chains and some are small. Yeasts in tube-like stems are also found.	Streptococci, yeast, <i>Bacillus subtilis</i> , staphylococci, Gram-positive bacilli, large streptobacilli. 15 tubes inoculated, 2 with pieces of ulcer. The latter contained streptococci together with other organisms. Of the remainder, 2 contained streptococci in pure culture, 1 contained streptococci and bacilli, and 7 were sterile.
Gas-tro-jejunal.	No bacteria demonstrable by Gram-Weigert method.	Streptococci, staphylococci, short Gram-positive bacillus. 11 tubes inoculated, 1 with piece of ulcer. Streptococci recovered in pure culture from 3 tubes and together with staphylococci in 2 tubes. 1 tube was sterile, 1 contained a short Gram-positive bacillus, and 4, including the tube inoculated with a piece of ulcer, contained staphylococci.

*Plain Blood Agar.*—Small discrete or fused colonies, gray by reflected light, and opaque and surrounded by a narrow green zone by transmitted light. No hemolysis.

From seven ulcers we recovered anhemolytic streptococci; streptococci were seen in cultures from the eighth but could not be isolated, and from the ninth no streptococci were recovered.

Yeasts were recovered from four ulcers. They were not identified, but grew luxuriantly aerobically on all the ordinary laboratory media. Morphologically slight variations in the strains were noted. They resembled morphologically the *Saccharomyces* described by Besson.<sup>3</sup>

### *Method of Inoculation.*

Recently isolated cultures were injected in most of the experiments, the exact generation being given in the table, "first generation" meaning the original culture. The material for inoculation was prepared as follows:

1. A 24 hour growth of streptococci was obtained either in deep tubes of glucose-serum-bouillon or on slants of glucose-serum-agar. About 20 cc. of the growth in bouillon were centrifuged, washed twice with normal saline solution, and the resulting sediment was taken up in 2 to 5 cc. of normal saline solution. The 24 hour growth from one to four glucose-serum-agar slants was similarly washed with normal saline solution and the sediment resuspended in salt solution.

2. A milky emulsion of a 24 hour culture of yeast on glucose-serum-agar was made in several cc. of normal saline solution.

The entire amount of suspension prepared was used for intravenous injection into the ear vein and from 1 to 2 cc. for injection into a branch of the gastric artery. For the gastric artery injection the animals were etherized and a laparotomy was performed under aseptic precautions. The stomach was delivered and a branch of the gastric artery, usually on the anterior wall, was injected with a very fine needle and Record syringe. The part supplied always blanched during injection, but the color immediately returned on withdrawal of the needle. Very little hemorrhage followed and this was quickly controlled by compression. The wounds in every animal healed *per primam* and none developed peritonitis. Subsequent investigation was made through a laparotomy wound, opening the stomach widely and inspecting the entire mucosa directly. These gastric incisions also healed promptly and in several animals the stomach was opened a second time without apparent injury.

<sup>3</sup> Besson, A., *Technique microbiologique et sérothérapique*, Paris, 5th edition, 1911.

*Animal Experiments.*

Rabbits and cats were used in our experiments. Most of the rabbits were injected intravenously with streptococci, but in some the injections of either streptococci or yeast were made directly into a branch of the gastric artery. Streptococci were injected into a branch of the gastric artery in two cats and a yeast in the other two.

Table II gives in a condensed form the experiments and their results.<sup>4</sup>

*Gastric Lesions in Rabbits Following Intravenous Injection of Streptococci.*

Thirty rabbits were injected intravenously with streptococci. Gastric lesions developed in four (13.3 per cent).

*Rabbit A III.*—Killed 2 days after injection.

*Stomach.*—Over the anterior wall of the stomach near the cardia there are three pale, almost circular areas seen on the serosa. One of these is at the termination of a group of blood vessels springing from the lesser curvature. Beneath these areas are what appear to be defects in the mucosa, from 2 to 10 mm. in width. The edges are abrupt and irregular and one cannot tell whether the base of the defects is covered by the mucosa or not.

*Microscopical Examination.*—The defects extend almost through the mucosa, with slight inflammatory reaction about them. Gram-Weigert stain shows various types of bacilli and cocci along the entire surface of the mucosa and defect. The same organisms occur in the tissues just beneath the surface, but at no greater depth at the site of the defect than elsewhere. No predominance of cocci.

*Rabbit C III.*—Killed 1 day after injection.

*Stomach.*—The anterior wall of the cardiac region is bright red. The mucosa beneath this is also bright red. No defects found.

*Microscopical Examination.*—(Two blocks.) Section 1. From non-hemorrhagic area. Tissues are normal. There are no bacteria in the mucosa, but on the surface are found the large Gram-positive bacilli that occur on the surface of all stomach sections.

Section 2. From hemorrhagic area. There is an engorgement of the capillaries in the mucosa just beneath the surface, and slight extravasation. No bacteria in this area. At the other end of the section, which includes normal tissue, just beneath the surface of the mucosa, there is a very large clump of Gram-positive cocci, some of them in chains, but mostly occurring as diplococci or

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<sup>4</sup> A number of experiments were performed with organisms recovered from other gastric ulcer cases, which we hope to embody in a later report.

TABLE II.  
*Stomach Lesions.*

Ulcer.	Rabbit.	Days animal lived after injection.	Generation of streptococcus or yeast injected.	Intra-venous injection.	Gastric artery injection.	After intravenous injection.	After gastric artery injection.	Heart lesions.	Cultures of urine, bile, and heart's blood.
A	I	1	Streptococcus.						
	II	4	2	+					
	III	2	2	+					
	IV	10	2	+					
C	I	2	3	+		Defect.		Few hemorrhages. Hemorrhages.	Negative.
	II	3	3	+				Endocardial hemorrhages. Hemorrhages.	"
	III	1 (Killed.)	4	+		Hemorrhages.		"	Streptococci from blood; urine and bile negative.*
	IV	3	4		+		Abcesses and small defects.	Gray streak in septum.	Streptococci and staphylococci in blood; urine and bile negative.†
D	II 1	3	3 (From Rabbit C II.)	+				Pink and gray nodules in endocardium, epicardium, and valves.	Negative.
	II 2	6	3 (From Rabbit C II.)	+					"
	I	5	1	+					Streptococci from heart's blood.
	II III	3 2	1 3	++				Gray streak in septum.	Negative. Streptococci from blood and urine.‡



E	IV	4	3				
E	I	Died immediately. ‡	Original bouillon culture. Streptococci seen; not isolated.	+	Gray streaks in heart.		
	II	1 (Died.)		+	Cocci found microscopically in blood vessels; no lesions.		Staphylococci and bacilli from heart's blood. **
	III	1 "		+			
G	I	2	1	+	Hemorrhages.	Negative.	
	II	7	1	+		"	
	III	7	1	+		"	
	IV	2	2	+		"	
	V	14	2	+	Mottled appearance.		Streptococci from heart's blood.*
	VI	3	2	+		Negative.	
J	VII	5	3		Defect. Minute hemorrhages.		
	VIII	4 (Died.)	3				
	I	7	1	+	Defect.		"
	II	4	1	+			"
	III	7	4				"
	IV	4	4	+			"
	V	7	4				
	VI	7	4	+	Hemorrhage in tricuspid valve.		"

\* Gray streaks in skeletal muscles. † Hemorrhages in intestines and appendix. ‡ Tissues throughout body reddened. § Died with symptoms of anaphylactic shock. || Hemorrhages in intestines. \*\* Tissues congested.

TABLE II—*Concluded.*

Ulcer.	Rabbit.	Days animal lived after injection.	Generation of streptococcus or yeast injected.	Intra-venous injection.	Gastric artery injection.	After intravenous injection.	After gastric artery injection.	Heart lesions.	Cultures of urine, bile, and heart's blood.
M	I	7	Streptococcus.						
	II	16	1		+		Defect.		Negative.
	III	7	1 (Killed culture.)		+		Minute hemorrhages.		
	IV	2	1	+					
	V	11	1	+					
Gastro-jejunal ulcer.	I	2	3	+					Streptococci from heart's blood.
	II	5	3	+					
	38			30	8	4 (13.3 per cent.)	6 (75 per cent.)	16	
L			Yeast.						
	I	5	1	+					
	II	7	3		+				
	III	1	3	+		Hemorrhage.		Hemorrhages.	
	IV	7	3	+		"			
Summary	4			3	1	2 (66 per cent.)	0	1	



as bacillary forms. There is no inflammatory reaction found around this area and only a slight, almost insignificant amount of extravasation of red blood cells.

*Rabbit G IV.*—Killed 2 days after injection.

*Stomach.*—On the anterior wall of the pyloric antrum, near the lesser curvature are two hemorrhagic areas in the mucosa, 2 by 4 mm. in size, and a few mm. from one another. There is a superficial defect over these areas and the mucous membrane around them for a distance of 2 mm. is slightly reddened.

*Microscopical Examination.*—Serial sections of superficial defect. Gram-Weigert stain. A V-shaped defect, half way through the mucosa, in the midst of the hemorrhagic area. Very large bacilli are invading the defect from the surface. No streptococci. No bacteria in any of the blood vessels.

*Rabbit G VI.*—Animal became sick and was killed 3 days after injection.

*Stomach.*—On the mucosa on the anterior wall of the pyloric antrum there is a superficial hemorrhagic defect, 2 by 4 mm. The mucosa around it is reddened, and adherent to it is blood-stained mucus.

*Microscopical Examination.*—A considerable area of mucosa shows slight hemorrhagic infiltration, with hyaline thrombosis in a few of the superficial blood vessels and moderate degeneration of the superficial third of the mucosa. Just beneath the center of this area are several arteries and veins in the submucosa which show slight inflammation of their walls but no change in the lumen.

*Serial Sections.*—Gram-Weigert stain. No cocci in any of the vessels going to the mucosa. No bacteria except surface bacteria of various types.

### *Gastric Lesions in Rabbits Following the Injection of a Branch of the Gastric Artery with Streptococci.*

In eight rabbits a branch of the gastric artery was injected with streptococci, according to the method described above. Gastric lesions developed in six (75 per cent).

*Rabbit C IV.*—Killed 3 days after injection.

The peritoneal cavity contains a small amount of fluid and clotted blood, and the omentum and portions of the stomach are infiltrated with blood. Omentum adherent over stomach. Serosa over injected area is pale, and shows beneath it a few pin-head, cream-colored areas, which look like miliary abscesses. Mucosa over these areas shows small hemorrhagic patches with slight defects in the mucosa, as well as miliary, cream-colored areas similar to the above.

*Microscopical Examination.*—There are several large hemorrhagic areas in the serosa and collections of leukocytes almost to the degree of abscess formation. There are about half a dozen depressions along the surface of the mucosa which are V-shaped, and about these depressions is a slight hemorrhagic infiltration with partial degeneration (?) of the structures. These areas reach half way through the mucosa. Over most of these depressions the surface epithelium is

intact but slightly degenerated and there is a slight fibrinous exudate adherent to the surface.

Gram-Weigert stain. Upon the mucosa over the hemorrhagic and purulent areas are a great number of bacteria, most of them large bacilli; no bacteria are found in the hemorrhagic and purulent areas in the mucosa. Bacteria are present in the serosa and correspond to those on the surface of the stomach. These are probably secondary invaders.

*Rabbit G VII.*—Killed 5 days after injection.

*Stomach.*—The branch of artery injected is identified by a small hemorrhagic spot at the site of injection. Distal to this the vessel seems empty. On the serous coat, in the portion directly supplied by the injected artery, an area, 1.5 by 3 cm., shows beneath the serosa a thin layer of thick, cream-colored pus. Beneath this is seen, from the mucosal side, an irregular, punched out defect, 1 by 0.5 cm., with raised, edematous edges, and an area of edema around it, 2 cm. in width. The base of the defect is formed by a very thin, transparent portion of the wall of the stomach. About 2 cm. from the defect is a hemorrhage in the mucosa, 2 mm. wide. The remainder of the stomach is normal.

*Microscopical Examination.*—The base of the defect shows degeneration and necrosis almost as far as the serosa. The edge of the defect is fairly sharp and shows a similar condition of degeneration.

Gram-Weigert stain. In the depth of the defect, just beneath the serosa and reaching for a short distance into the muscularis, are found solid clumps of streptococci filling the capillaries and small blood vessels. Between these, scattered throughout the tissues, are diffuse collections of streptococci. On and immediately beneath the necrotic surface of the defect are large clumps of bacilli. Individual bacilli also lie scattered between the clumps.

On cross section of a portion of the stomach with arteries near the defect, one of the arteries is seen to be filled with an organizing thrombus; the other arteries are normal. There are no bacteria in the thrombus.

*Rabbit G VIII.*—Died 4 days after injection.

*Stomach.*—About one-third filled with food. Delicate adhesions between the cardiac half of stomach and surrounding viscera. Over the cardiac half of the stomach, especially near the greater curvature, the mucosa shows numerous minute hemorrhages. No defects.

Microscopical examination was not made.

*Rabbit J III.*—Killed 7 days after injection.

*Operation.*—Attempt to make injection into a branch of the gastric artery near the cardiac end of the stomach on the anterior wall failed. Injection was made into another blood vessel on the anterior wall of the stomach, though it could not be determined whether it was a vein or an artery. No blanching of the portion of the stomach injected. Previous attempt at injection resulted in a large hematoma at the lesser curvature of the stomach. Two large branches of the gastric artery on the posterior wall of the stomach were injured in delivery of the stomach. Animal returned to cage in good condition.

*Stomach.*—Hematoma along the lesser curvature. About the vessel on the anterior wall which was injected there is an area about 1 by 0.5 cm. showing hemorrhages and a small amount of thick pus beneath the serosa. On the posterior wall of the fundus near the greater curvature, corresponding to the area supplied by the injured arteries, the stomach is adherent in two places to one of the lobes of the liver. The mucosa of the stomach immediately beneath these adherent areas shows an irregular defect 0.5 cm. in width which extends half way down through the gastric wall. The base of the defect is reddened; the edge is edematous and slightly reddened. Beneath the hemorrhagic areas, on the anterior wall of the stomach where the injection was made, there are no changes in the mucosa. The stomach is otherwise negative.

*Microscopical Examination.*—Defect on posterior wall. Superficial defect half way through the mucosa with degenerative changes, slight hemorrhages, etc. One large vein in the submucosa shows beginning mural thrombosis. No streptococci or other bacteria in the depth of the defect. Various types of bacteria present on the surface of the normal portion of the stomach and on the surface of the defect. Adhesions over the serosa contain no bacteria. Gastric arteries supplying the area of the defect are normal.

Gram-Weigert stain. Usual types of bacteria on surface of defect and neighboring normal mucosa, none in tissues except a few surface type organisms in the hemorrhagic area of the serosa.

The serosa of lesions on the anterior wall shows edema and slight hemorrhagic and leukocytic infiltration. Vessels and mucosa are normal. Gram-Weigert stain. Usual surface bacteria are present, but no organisms are found in the tissues.

*Rabbit M I.*—Killed 7 days after injection.

*Stomach.*—On the serous surface of the anterior wall of the fundus, in the center of the region supplied by the injected artery, there is a creamy yellow area, 1 by 2 cm., where the omentum is adherent. There are other smaller areas of thickening of serosa. Beneath the large area, practically the entire thickness of the stomach wall from the mucosa outward is necrotic, forming a defect with hemorrhagic edge and necrotic base. The branch of artery injected is in good condition.

*Microscopical Examination.*—There is a defect of the mucosa. Beneath the defect the gastric wall shows large masses of pus cells, edema, congestion, and degeneration of tissues, amounting almost to necrosis in places. No thrombi in veins or arteries.

Gram-Weigert stain. There are masses of cocci in the inflamed and degenerating area and similar masses in the overhanging edge of the defect apparently lying in the capillaries or lymphatics.

*Rabbit M III.*—Killed 7 days after injection with killed culture of streptococci.

*Stomach.*—Minute scar at the point of injection of the artery. Mucosa of fundus, near the greater curvature, shows a few minute hemorrhages but no defect over them.

*Microscopical Examination.*—Congestion of superficial capillaries. No hemorrhages. Gram-Weigert stain. No cocci, or other bacteria, except a few usual types on the surface.

*Gastric Lesions in Cats Following the Injection of a Branch of the Gastric Artery with Streptococci.*

In two cats a branch of the gastric artery was injected with streptococci. Both animals developed defects in the gastric mucosa, which soon began to heal and were observed to have healed completely in 33 days.

*Cat J I.*—Cat well 9 days after injection.

*Stomach Operation.*—Explored under ether anesthesia. Few adhesions found between the anterior wall of the stomach and the omentum, and between the posterior wall and the posterior wall of the lesser peritoneal sac. Along the lesser curvature there is an indurated area about 1 cm. wide in the region supplied by the injured artery. Beneath this is seen a punched out area in the mucosa that may be either a shallow erosion or healing defect. Stomach otherwise negative. The incision in the stomach, which was parallel to the greater curvature and 1 cm. from it on the anterior wall, was closed with a double row of sutures. 33 days after injection the abdomen was opened under ether anesthesia. No sign of defect found on serous or mucous coats of stomach. Animal killed.

*Cat J II.*—18 days after injection. Cat has remained entirely well and well nourished.

*Operation.*—Performed under ether anesthesia. Stomach free and nowhere adherent. At the lesser curvature, where the injected artery enters the stomach, there is an indurated area involving the entire wall of the stomach, 1.5 cm. wide and about the normal stomach wall in thickness. The stomach was opened on the anterior wall parallel to the greater curvature for a distance of 4 cm., 1.5 cm. from the greater curvature and the same distance from the indurated area. Beneath this indurated area is a shallow, irregularly round defect in the mucosa with indurated margins. The edge of the defect is formed by a bright red line, apparently of granulations, as though it were healing. Stomach closed. Animal returned to cage in good condition. 33 days after the injection the cat was again etherized. Slight puckered scar of serosa over the site of the previous defect. Mucosa at corresponding point shows slightly puckered area, entirely healed. Animal killed.

*Microscopical Examination.*—The mucosa is completely regenerated over the site of the ulcer. It is made up of glands which are slightly atypical. A slight defect in the submucosa has also been repaired. Gram-Weigert stain shows no bacteria.

*Gastric Lesions in Rabbits Following Injection with Yeast.*

Three rabbits were injected intravenously. Two of these animals developed minute hemorrhages in the gastric mucosa.

*Rabbit L III.*—Died 12 hours after injection.

*Stomach.*—Many minute hemorrhages in mucosa.

*Microscopical Examination.*—Negative; no yeast.

*Rabbit L IV.*—Killed 7 days after injection.

*Stomach.*—Few minute hemorrhages in mucosa, not examined microscopically.

In addition a branch of the gastric artery was injected in one rabbit (L II). The animal was killed on the 7th day and showed no lesions at autopsy.

*Gastric Lesions in Cats Following Injection of Yeast into a Branch of the Gastric Artery.*

Two cats (L I, M I) were injected. The animals died on the 12th and 13th days respectively, and no lesions were found at autopsy.

*Summary of Cardiac Lesions.*

Of the thirty rabbits injected intravenously with streptococci, fourteen (47 per cent) developed cardiac lesions. In two, minute vegetations containing streptococci were present. The remainder showed either petechial hemorrhages or the gross appearance of myocarditis, and practically all of these hearts contained microscopical lesions of the Bracht and Wachter type.<sup>5</sup>

Of the eight rabbits in which a branch of the gastric artery was injected two (25 per cent) developed minute hemorrhages in the endocardium.

Of the three rabbits injected intravenously with yeast one showed hemorrhages in the endocardium.

## DISCUSSION.

As a result of the above studies it is impossible to decide definitely whether or not the gastric lesions produced by the injection of streptococci are to be considered ulcers. The superficiality of the rabbit lesions following the injection by the intravenous route as well as the entire absence of inflammatory reaction in the deeper gastric

<sup>5</sup> Thalhimier, W., and Rothschild, M. A., *Jour. Exper. Med.*, 1914, xix, 429.



tissues leads us to the belief that these defects are certainly not analogous to the chronic gastric ulcer seen in the human stomach. The promptness of healing of the embolic lesions in the cats tends to strengthen this conclusion. This point will be discussed more fully below.

Apparently striking results were obtained in Cats J I and J II, where the injection of streptococci into a branch of the gastric artery resulted in the formation of shallow, indurated defects that might be considered ulcers. However, these defects were found to be healing on the 10th and 18th days respectively, and were completely healed on the 33rd day.

It has been demonstrated by Bolton,<sup>6</sup> Wilensky and Geist,<sup>7</sup> and others that defects produced mechanically in the gastric mucosa and muscularis of cats heal in 2 to 4 weeks. In the defects produced in the rabbits by injection into a branch of the gastric artery streptococci were found in great numbers in the tissue about the lesions. By analogy it may be assumed, even in the absence of microscopic proof, that streptococci were also present at some time in the lesions in the cats. The embolic lesions in the stomachs of the cats in our series healed spontaneously within approximately the same length of time as those mechanically produced. It is evident, therefore, that in these instances the injected streptococci failed to retard the process of healing.

The constant presence of an anhemolytic streptococcus in human gastric ulcers might be adduced as an argument in favor of the part played by this organism as the cause of the chronicity of the lesion. If streptococci could be demonstrated in considerable numbers in the depth of human gastric ulcer this conception would gain a firmer basis. Rosenow<sup>1</sup> has found streptococci in all the coats of the stomach, some of the organisms even lying just beneath the serosa. No mention is made of the number of organisms in this position. In our series of ulcers, however, despite the examination of many sections, the streptococci were found only on or beneath the surface of the defect.

<sup>6</sup> Bolton, C., *Jour. Path. and Bacteriol.*, 1915, **xx**, 133.

<sup>7</sup> Wilensky, A. O., and Geist, S. H., *Jour. Am. Med. Assn.*, 1916, **lxvi**, 1382.

The case of gastrojejunal ulcer described above seems of importance in this connection. This ulcer might readily be considered as due primarily to purely mechanical factors. Streptococci were recovered culturally from emulsified pieces of tissue. Although the histological picture was identical with that of the other ulcers in the series the most careful search failed to show the presence of streptococci on the surface of the lesion or in the tissues about it. One must consider the possibility that in this case the streptococci were directly deposited upon a preexisting mechanical defect and were not the essential causative factor in the formation of the ulcer.<sup>8</sup>

A possible explanation of the occurrence of streptococci in gastric ulcers may be found in some observations of Cushing and Livingood.<sup>9</sup> These investigators found that whereas there is a more or less definite bacterial flora of the ileum and large intestine, this is not true of the upper portion of the alimentary tract. The stomach and the duodenum and at times also the upper jejunum become free or practically free of bacteria after a certain period of fasting, whereas during and shortly after a meal great numbers of organisms of different types are found. These are greatly reduced in number when sterile food alone is taken. Cushing and Livingood, therefore, concluded that the normal stomach has no definite bacterial flora but that this is dependent upon the bacteria taken with the food.

<sup>8</sup> Since this article was completed we have studied another gastrojejunal ulcer that had the gross and microscopic appearance of a chronic gastric ulcer. No bacteria were found with the Gram-Weigert stain either upon the surface of the lesion or in the underlying tissues. Anhemolytic streptococci were isolated from several tubes of glucose-serum-agar inoculated with the emulsified ulcer. Other bacteria similar to those described in the body of the paper were also recovered.

Six rabbits were injected intravenously with the second generation of the streptococcus and were killed at periods varying from 2 to 10 days. At autopsy there were small hemorrhages in the duodenal mucosa of one rabbit, hemorrhages in the tricuspid valve in two others, and minute tricuspid vegetations in a fourth. The structure of these vegetations on microscopical examination as well as the absence of streptococci in smears and sections indicates that the injected organisms were not the cause of the lesion. No other lesions were found in these four animals, and in the remaining two the autopsy was entirely negative. No streptococci were found either culturally or microscopically in the hemorrhagic area in the duodenum.

<sup>9</sup> Cushing, H., and Livingood, L. E., *Johns Hopkins Hosp. Rep.*, 1900, ix, 543.

Cushing and Livingood showed that neither the acidity nor the digestive ferments of the gastric secretion have marked lethal effect on the bacteria in the stomach. They believe that the stomach becomes aseptic chiefly through the mechanical removal of organisms in the passage of the ingesta, and that bacteria cannot adhere to normal gastric mucosa nor do they enter or remain in the glandular crypts. Whether bacteria can adhere to the surface or penetrate into the depths of defects in the mucosa remains to be determined. These writers further state that there were two organisms from which the stomach freed itself with difficulty, and which were found in a number of examinations many hours after the intake of food. These organisms were a minute Gram-positive diplococcus and yeast. The same forms were found almost constantly in our series of gastric ulcers both in sections and in cultures.

In the light of the work of Cushing and Livingood one must bear in mind the possibility that these organisms which are commonly found in the stomach and duodenum might remain upon or immediately beneath the degenerating lining of the ulcer because of favorable mechanical or structural conditions.

The nature of both the experimental gastric and cardiac lesions indicates that the streptococci recovered from the ulcers are of a low grade of pathogenicity, such as is usually found in the same type of organism isolated from other sources. Whether, having established themselves on the surface of a gastric ulcer in man, they are innocuous or manifest a low grade of pathogenicity tending to retard healing requires further investigation.

In addition to the streptococcus other organisms were invariably recovered from the inoculated tubes. No attempt was made to identify these strains culturally. The same types were usually identified microscopically in sections, proving that the isolated organisms other than streptococci were not accidental contaminations. Furthermore, these bacteria were quite as numerous in the sections as streptococci, and penetrated to the same depth in the ulcer. The types most frequently encountered were a yeast and a thick Gram-positive bacillus. Some of the isolated strains of yeast proved pathogenic for rabbits on intravenous inoculation. Our data are insufficient to warrant an expression of opinion as to the significance of these organisms.

## CONCLUSIONS.

It must be assumed that some cause is operative in certain cases preventing the healing of defects in the gastric mucosa and is inoperative in others. Even though anhemolytic streptococci are present in practically all gastric ulcers, we cannot convince ourselves that these organisms have been proven as yet to be the factor which either initiates the ulceration or prevents healing. Nevertheless, the constant presence of streptococci in this type of lesion is a suggestive fact and further experiments to determine their significance are being undertaken.

We wish to express our thanks to Dr. A. A. Berg, at whose suggestion this investigation was undertaken. We are further indebted to him for the material studied, for the privilege of reporting abstracts from his clinical records, and for other courtesies extended to us during the progress of this work.

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